# The Interactions of *Streptococcus pneumoniae* with the Dendritic Cells: Relevance for the Infection Outcome

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## Abstract

*Streptococcus pneumoniae*, a common bacterium of the human nasopharynx, can cause severe local infections such as otitis media and also life-threatening invasive diseases including pneumonia, sepsis and meningitis. Moreover, *S. pneumoniae* is the most common cause of community-acquired bacterial pneumonia. The respiratory mucosa of healthy individuals is scattered with sentinel cells including dendritic cells (DCs), which have an extraordinary capacity to interact with T and B cells and modulate their responses to invading pathogens. Because in the lung DCs are situated in close vicinity to alveolar epithelial cells, it appears conceivable that interactions between DCs and *S. pneumoniae* can strongly influence the outcome of the infection process. To clarify the specific contribution of pulmonary DCs to host defense against *S. pneumoniae*, bone marrow chimera mice were generated from CD11c-DTR transgenic mice since DCs of these mice can be transient depleted by administration of consecutive doses of diphtheria toxin (DT). Strikingly, depletion of DCs rendered those mice much more resistant to respiratory infection with *S. pneumoniae* than non-depleted animals. Thus, DC-depleted mice exhibited delayed bacterial systemic dissemination, significantly reduced bacterial loads in the infected organs and lower levels of serum inflammatory mediators than non-depleted animals. The superior resistance of DC-depleted mice to *S. pneumoniae* was associated with a better capacity to restrict pneumococci extrapulmonary dissemination. Furthermore, it was demonstrated that *S. pneumoniae* disseminated from the lungs into the regional lymph nodes in a cell-independent manner and that this direct way of dissemination was much more efficient in the presence of DCs. The possibility that *S. pneumoniae* might exploit the capacity of DCs to break tissue barriers to facilitate its extrapulmonary dissemination was then investigated. The obtained results provided evidence that *S. pneumoniae* induced the expression and activation of matrix metalloproteinase-9 (MMP-9) in cultured bone marrow-derived DCs. MMP-9 is a protease involved in the breakdown of extracellular matrix proteins and is critical for DC trafficking across extracellular matrix and basement membranes during the migration from the periphery to the lymph nodes. Furthermore, depletion of DCs correlated with significant decreased MMP-9 expression in the lungs of *S. pneumoniae*-infected mice.

In summary, the results of this thesis provide direct *in vivo* evidence that *S. pneumoniae* exploits DCs to disseminate from the initial site of infection. Therefore, modulation of DCs during pneumococcal pneumonia might provide an interesting means to prevent or delay extrapulmonary bacterial dissemination.

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# 1. Introduction

## 1.1. *Streptococcus pneumoniae*

### 1.1.1. Overview

*Streptococcus pneumoniae* (pneumococcus) is a gram-positive encapsulated bacterium that grows in pairs or chains and is facultatively anaerobic [1]. It was first isolated in 1881 simultaneously and independently by George M. Sternberg and Louis Pasteur [2]. Pneumococci are alpha hemolytic microorganisms which are often found as a normal commensal in the nasopharynx of healthy adults and children [3]. However, *S. pneumoniae* can also cause diseases that range in severity from life-threatening meningitis, septicemia, and pneumonia to more mild infections such as sinusitis and acute otitis media [4]. Although all age groups may be affected by *S. pneumoniae*, the highest rate of pneumococcal disease occurs in young children and in the elderly population [5]. In addition, persons affected by chronic conditions and immune deficiencies are at increased risk. *S. pneumoniae* is a major cause of morbidity and mortality. Thus, it has been estimated that more than 10 million children under 5 years of age are affected by pneumococcal disease every year [6].

*S. pneumoniae* is surrounded by a polysaccharide capsule that constitutes the basis for the serotyping classification of the bacterium in more than 90 serotypes [7]. The distribution of the different serotypes varies between geographic regions. While serotypes 6, 14, 19, and 23 are the most common serogroups worldwide, serotypes 1, 5, and 8 are the major cause of invasive pneumococcal disease in young children in developing countries [8]. Approximately 90% of the most frequent isolates belonging to 23 serotypes have been included in the 23-valent pneumococcal vaccine [9]. Following implementation of the pneumococcal conjugate vaccine, the incidence of invasive pneumococcal diseases has significantly declined in both children and adults [10, 11]. However, the emergence of serotypes not encompassed in the vaccine combination is a matter of concern [12]. In addition, antibiotic resistance among pneumococci has dramatically increased during the last three decades [13]. Therefore, diseases caused by *S. pneumoniae* remain a significant public health problem all over the world and renewed efforts are now being made to better understand the pathogenesis of pneumococcal diseases.

### 1.1.2. Virulence factors

As an extracellular bacterial pathogen, *S. pneumoniae* needs to attach to host cells, to replicate and to escape clearance by the host immune system. All these pathogenic functions are enabled by the coordinated expression of a wide array of virulence factors (Fig. 1). Thus, attachment of *S. pneumoniae* to eukaryotic cells is meditated by a diverse group of bacterial surface molecules including cell wall components, choline binding proteins, LPxTG proteins, lipoproteins, and non-classical adhesins such as surface associated enzymes [14]. Furthermore, *S. pneumoniae* has developed a number of strategies to avoid phagocytosis by the host cells. Among them, the capsule protects against phagocytic clearance by blocking the deposition and function of opsonins directed against cell surface antigens [15]. In addition, the pneumococcal capsule can reduce bacterial trapping by neutrophil extracellular traps (NETs) [16], killing by defensins [17], and clearance by mucus [18]. Other virulence factors such as the pneumococcal surface protein A (PspA), and the toxin pneumolysin are mainly involved in the induction of inflammation. The most relevant virulence factors expressed by *S. pneumoniae* are described below.

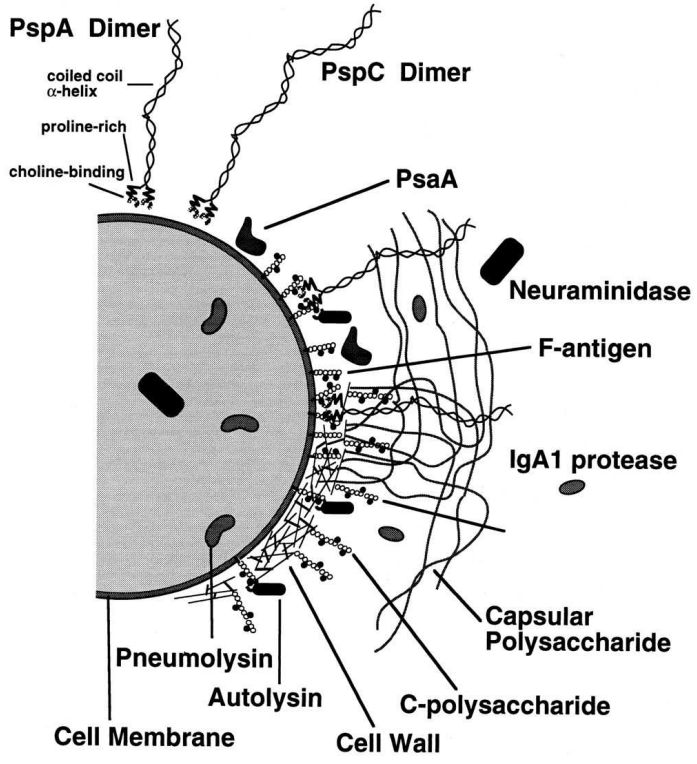


Figure 1: Schematic representation of the virulence factors expressed by *S. pneumoniae*. Adopted without modifications from Briles *et al*., 1998 [19].

1. Capsule

The capsule has long been considered to be the primary virulence factor of *S. pneumoniae* where its pathogenesis is underscored by the high level of attenuation exhibited by encapsulated strains [20, 21]. The pneumococcus capsule consists of chains of monosaccharides covalently attached to the outer surface of the cell wall peptidoglycan [22]. The chemical structure as well as the thickness of the capsule determines the ability of the different serotypes to survive in the bloodstream and possibly cause invasive disease [23]. More than 90 distinct capsular serotypes have been identified according to reactivity with polyclonal or monoclonal antibodies [7]. They differ in terms of activation of the alternative pathway of complement [24-26], deposition and degradation of complement components on the capsule [27, 28], resistance to phagocytosis [26, 29, 30], and the ability to induce antibody production [31]. Given its essential role in the pathogenesis of *S. pneumoniae* infection, capsular polysaccharides have been used in the 23-valent pneumococcal polysaccharide vaccine PNEUMOVAX 23® and the pediatric multivalent pneumococcal conjugated vaccines.

2. Cell wall

The cell wall of *S. pneumoniae* is composed of teichoic acids (TA) linked via a phosphodiester bond to the peptidoglycan backbone and lipoteichoic acids (LTA) linked to the cell membrane via a C-terminal fatty acyl group [32]. TA and LTA are potent pro-inflammatory constituents of *S. pneumoniae*. They are composed of repetitive oligosaccharide units conjugated to phosphorylcholine [33]. The inflammatory reaction associated with typical pneumococcal diseases such as otitis media, meningitis, and pneumonia can be mimicked in animals after injection of purified cell wall or its degradation products [34-36]. Furthermore, it has been shown that the pneumococcal cell wall activates the alternative pathway *in vitro* [37, 38] and that the activation of the complement system may be involved in the induction of lung and central nervous system inflammation by *S. pneumoniae in vivo* [36, 39]. Other central mediators of the inflammatory process induced by the pneumococcal cell wall are interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α). Purified cell wall and TA are powerful inducers (even stronger than endotoxin) of IL-1 production by human peripheral monocytes [40] whereas LTA is able to trigger TNF-α release [41]. Furthermore, the muramyl dipeptide, a structural component of the pneumococcal peptidoglycan, can also induce production of cytokine IL-1 [42].

3. Pneumolysin

Pneumolysin is a cytoplasmatic enzyme that belongs to the family of the thiol-activated toxins [43]. It is produced as a 52 kDa soluble protein that is released upon bacterial lysis induced by autolysin. At high concentrations, it oligomerizes in the membrane of target cells to form a large ring-shaped transmembrane pore. The pore is 260 Å in diameter and is composed of approximately 40 monomeric subunits [44]. At lower concentrations, the toxin has several effects on different cell types, including stimulation of inflammatory cytokines by human monocytes [45], inhibition of the cilia beating on human respiratory epithelial cells, disruption of the monolayers in cultured respiratory epithelium [46, 47], induction of apoptosis of murine dendritic cells [48] and neuronal cells [49-51], reduction of the bactericidal activity as well as migration of neutrophils [52], and inhibition of lymphocyte proliferation and antibody synthesis [53]. Furthermore, pneumolysin has been reported to activate the classical complement pathway [54]. Recognition of pneumolysin by immune cells is mediated by Toll-like receptor 4 [55]. The importance of pneumolysin for *S. pneumoniae* infection is highlighted by the lower level of virulence exhibited by pneumolysin-negative mutants in comparison with their parental strains [56] and, by the extended survival of pneumolysin-immunized mice after challenge with pneumococci of different serotypes [57-59].
Pneumolysin is found in virtually all pneumococcal isolates and its amino acid sequence is well conserved, although a small number of variants have been described [60, 61].

4. Pneumococcal surface protein A

Pneumococcal surface protein A (PspA) is a surface-exposed protein, which varies in structure and antigenicity between the different pneumococcal strains. PspA is expressed by most clinical isolates [62] and it has a unique structure with similarities to other surface proteins in gram-positive bacteria (e.g. M proteins of group A streptococci) [63]. PspA has been reported to inhibit C3-mediated opsonization of *S. pneumoniae* [64] and disruption of the *pspA* gene resulted in reduction or even loss of bacterial virulence [65, 66]. Furthermore, passive immunization of mice with polyclonal or monoclonal antibodies raised against PspA or active immunization with either recombinant PspA or its N-terminal fragment has been shown to be protective against challenge with pneumococci of various capsular serotypes [67-69].

5. Autolysin (LytA)

The autolysin LytA is an N-acetylmuramic acid-L-alanine amidase that is located in the cell envelope of the pneumococcus. This enzyme is involved in the process of cell division by cleaving the peptidoglycan in the cell wall. The name autolysin is due to the fact that it induces lysis of pneumococci under certain conditions such as nutrient starvation during the stationary phase of growth [70].

Immunization of mice with autolysin confers some level of protection against challenge with a virulent wild-type strain [71]. In addition, LytA-deficient *S. pneumoniae* mutant strains were shown to have reduced virulence in murine models of pneumonia and bacteremia [71-74]. The functional activity of autolysin seems to be mediated by the release of cytoplasmic bacterial proteins including pneumolysin as well as cell wall constituents [75].

6. Adhesins

For successful colonization and establishment of infection *S. pneumoniae* must first attach to the epithelial surface. This process is mediated by the expression of adhesins that target host components including lipoproteins, choline-binding proteins (CBPs), proteins with an LPxTG motif and non-classic surface proteins. In particular, CBPs and non-classic surface proteins that lack a leader peptide or membrane-anchor motif display adhesive functions [14, 76]. Proteins with an LPxTG motif have rather enzymatic activities which unveil host cell receptors and aid the bacterial adherence to the host cells [76]. Unlike LPxTG proteins, CBPs are non-covalently linked to the phosphorylcholine of the pneumococcal cell wall. Interestingly, phosphorylcholine itself has been shown to function as an adhesin by recognizing the platelet-activating factor receptor (PAFR) of host cells [77, 78]. Among CBPs, the major pneumococcal adhesin in the nasopharyngeal cavity is the multifunctional pneumococcal surface protein C (PspC). PspC interacts with the ectodomain of the polymeric immunoglobulin receptor in a human-specific manner [79-81] and with the innate immune components factor H and C3 which promotes pneumococcal adherence as well as immune evasion [82-84].

In addition, SlrA (streptococcal lipoprotein rotamase A) and PpmA (putative proteinase maturation protein A) have been shown to be involved in pneumococcal colonization since deficiency in either of these lipoproteins reduced bacterial adherence to and internalization by epithelial and endothelial cells and resulted in rapid bacterial clearance from the nasopharynx in a murine pneumonia model [85, 86]. The pneumococcal surface adhesin A (PsaA) is part of an ABC transporter complex for manganese and thought to interact with E-cadherin [87].

Mutants lacking PsaA exhibited reduced adherence capacity to human epithelial cells *in vitro* [88] as well as a reduced ability to colonize the nasopharynx *in vivo* [89].

In order to gain access to the submucosa and the bloodstream, pneumococci have been reported to breach barriers including the epithelium and endothelium as well as the extracellular matrix (ECM) by interacting with plasminogen and fibronectin [90, 91]. Fibronectin-binding was reported for PavA (pneumococcal adherence and virulence factor A), whereas PavB and PfbA (plasmin- and fibronectin-binding protein A) are able to bind both fibronectin and plasminogen [92]. In addition, the glycolytic enzymes α-enolase, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and the choline-binding protein E (CBPE) have also been shown to bind human plasminogen [91, 93, 94]. After recruitment of host plasminogen to the bacterial surface the zymogen is converted to the serine protease plasmin which promotes degradation of various ECM compounds such as fibrin and laminin [76]. In addition, pneumococci have been shown to produce proteases such as the zinc metalloproteinase C (ZmpC) that activates human matrix metalloproteinase 9 (MMP-9) [95]. Thus, ZmpC can contribute to tissue invasion, since MMP-9 cleaves gelatin and collagen of the ECM [96].

### 1.1.3. Antibiotic resistance of *S. pneumoniae*

The prevalence of antibiotic-resistant *S. pneumoniae* has increased worldwide. Since the first pneumococcal isolate resistant to penicillin was isolated from a patient in Australia in 1967 [97], penicillin-resistant pneumococci have spread all over the world [98, 99]. In some parts of Latin America the rates of penicillin resistance among the pneumococci are as high as 60% and in some countries in Asia as high as 80% [100, 101]. Resistance to penicillin may occur in combination with resistance to other antimicrobial agents [102]. Pneumococci resistant to more than two separate classes of antibiotics are considered to be multi-resistant. Thus, strains of *S. pneumoniae* exhibiting multiple antibiotic resistance including penicillin, tetracycline, erythromycin, clindamycin, trimethoprim-sulfamethoxazole, and chloramphenicol were initially reported in 1977 in South Africa [103] and subsequently in Europe [4, 104, 105] as well as in the United States [106]. Penicillin-resistant and multiple resistant strains belonged predominantly to serotypes 6, 19, 14, and 23 [107-109], with 23F being the most associated with multi-resistance [110, 111]. Pneumococcal resistance to essential antimicrobials is a serious and rapidly increasing problem worldwide.

### 1.1.4. Infections caused by *S. pneumoniae*

*S. pneumoniae* is a frequent colonizer of the human nasopharynx. From this location, the bacterium can spread to the upper and lower respiratory tract and can cause non-invasive diseases such as otitis media or non-bacteremic pneumonia, which is an infection of the lower respiratory tract without detectable spread of bacteria to the bloodstream. However, *S. pneumoniae* can also cause severe invasive diseases including bacteremia (bacterial infection of the blood), meningitis and bacteremic pneumonia, which is an inflammation of the lungs, with pneumococci also reaching the bloodstream [4]. Pneumococci are transmitted from person to person via respiratory droplets. Infection usually begins with the upper respiratory tract and then travels into the lungs. Pneumonia occurs when the bacteria find their way deep into the lungs, to the alveoli. Once in the alveoli, *S. pneumoniae* begins to grow and multiply resulting in the extravasation of inflammatory cells and fluids at the site of infection into the alveoli. This inflammation of the lungs is known as pneumonia.

The incidence of pneumococcal pneumonia is highest in children under 2 years of age, in people over 60 years of age and people with immunodeficiency [105, 106]. Rudan *et al*. reported that most episodes (> 95%) of early childhood pneumonia in children aged 0 - 4 years occur in developing countries, at an incidence rate of 0.28 episodes per year [112]. The incidence of hospitalized pneumonia in adults has been estimated at around 2.75 - 2.96 per 1000 population, with a steeply cumulative incidence with increasing age, and in-hospital mortality of around 14% [113].

*S. pneumoniae* can also spread from the upper respiratory tract to the middle ear, where it causes inflammation followed by accumulation of middle ear secretions. Inflammation or infection of the middle ear has been termed acute otitis media (AOM). AOM is one of the most frequent bacterial infections in children aged < 5 years. *S. pneumoniae* causes 30% - 50% of all cases of AOM worldwide [114].

From the upper respiratory tract, the lungs or the middle ear pneumococci can invade the bloodstream and move across the meninges causing meningitis [115]. The meninges are the membranes that surround and protect the brain and spinal cord. They are filled with liquid where *S. pneumoniae* can freely multiply. The risk of developing pneumococcal meningitis is highest among young children, older adults, persons with chronic illnesses, and immunocompromised individuals [116, 117]. Even with advances in medical care, the case-fatality rate has a range of 16% - 37% in adults and 1% - 3% in children [118]. Those who survive the disease have a 30% - 52% risk of neurological sequelae [119, 120].

### 1.1.5. Pneumococcal vaccines

The first attempt to vaccinate against *S. pneumoniae* was performed using whole killed pneumococci. However, this approach failed due to the adverse side effects caused by the high amounts of inocula required for inducing an immune response. The breakthrough in pneumococcal vaccine took place in the 1930s, after the development of pneumococcal serotyping and the demonstration of the immunogenicity of purified capsular polysaccharides. Today, more than 90 different serotypes based on differences in their polysaccharide capsule have been described [7]. The prevalence of the different serotypes is unequal, with the majority of pneumococcal diseases caused by around 20 serotypes. The predominant serotype varies with age, geography and site of infection [121]. Currently two types of vaccines, the pneumococcal polysaccharide vaccine (PPV) and the pneumococcal polysaccharide conjugated vaccine (PVC), have been approved for the prevention of pneumococcal disease. The current available pneumococcal polysaccharide vaccine is Pneumovax®23 (Merck), which is comprised of purified polysaccharides of 23 different serotypes. This 23-valent vaccine contains 23 capsular polysaccharide antigen serotypes that are responsible for about 90% of invasive pneumococcal infections [122-124]. However, children under two years of age and immunodeficient individuals respond poorly to this vaccine, thus reducing the protective value of the vaccine in two major target populations [125, 126]. In addition, the vaccine has no significant effect on nasopharyngeal carriage. To overcome these limitations, a new generation of protein-polysaccharide pneumococcal vaccines based on the conjugation of selected capsular polysaccharide to a protein carrier has been generated. The protein carriers induce a T cell-dependent immune response to the polysaccharides, leading to immunological memory and boosting upon repeated injection. A 7-valent conjugated vaccine that targets the most common serotypes of pneumococci causing diseases in children is currently available [127]. Because the number of other pneumococcal serotypes is becoming increasingly more common in children, a pneumococcal conjugated vaccine containing six additional polysaccharides has been generated (13-valent) [128]. Searching for new pneumococcal vaccines is still an ongoing issue because although the rates of invasive pneumococcal infections caused by the capsular serotypes included in the conjugated vaccine have declined, rates of invasive diseases caused by non-vaccine serotypes have increased in many places [12]. This phenomenon is known as “serotype replacement”.

## 1.2. The host innate immune response to *S. pneumoniae*

### 1.2.1. Immune recognition of *S. pneumoniae*

The innate host defense mechanisms against *S. pneumoniae* include opsonization, activation of complement and coagulation cascades, phagocytosis and activation of pro-inflammatory signaling pathways [129]. The recognition of invading pneumococci by the host innate immune system is mediated by pathogen recognition receptors (PRRs). The most important PRRs for *S. pneumoniae* are the members of the toll-like receptor (TLR) family, particularly the TLR2, TLR4, and TLR9 [55, 130, 131]. TLR2 has been reported to recognize lipoteichoic acid and cell wall peptidoglycan [130, 132, 133]. In a meningitis model, mice deficient in TLR2 expression displayed increased disease severity and decreased survival times compared with wild-type counterparts [134]. On the other hand, during pneumococcal pneumonia TLR2-deficient mice showed no differences in bacterial clearance and morbidity compared to the wild-type animals, although they exhibited reduced pulmonary inflammation [135]. Furthermore, van Rossum *et al*. [136] demonstrated in a nasopharyngeal colonization model reduced clearance of pneumococci after intranasal challenge, providing evidence that TLR2 also plays a role during carriage of *S. pneumoniae*. A protective role in pneumococcal pneumonia also has been demonstrated for TLR4 [137], although the protective effect is restricted to airway surfaces since the absence of TLR4 had no effect on survival rates and blood bacterial counts after intravenous infection of mice [138]. The bacterial ligand for TLR4 seems to be pneumolysin [55, 139]. Pneumolysin as well as DNA are released by *S. pneumoniae* that undergo apoptosis. Unmethylated cytosine-phosphate-guanosine (CpG) motifs in bacterial DNA are recognized by TLR9 [140]. Although TLR9 has been shown to be redundant in the control of nasopharyngeal colonization and pneumococcal meningitis [131, 141], TLR9-deficient mice exhibited reduced survival rates and enhanced bacterial loads in the lungs during pneumococcal pneumonia [131]. Upon activation, most PRRs induce the production of inflammatory mediators, including the cytokines TNF-α, IL-1ß, IL-6 and IFN-γ (interferon-gamma).

Other receptors expressed by macrophages critical for host defense against *S. pneumoniae* include the C-type lectin receptor SIGN-R1 (specific intracellular adhesion molecule-3 grabbing nonintegrin homolog-related 1) and the scavenger receptor MARCO (macrophage receptor with collagenous structure). Mice lacking either SIGN-R1 or MARCO exhibited increased susceptibility to pneumococcal pneumonia [142, 143].

### 1.2.2. The role of macrophages and neutrophils

The control of *S. pneumoniae* by the host immune system is critically dependent on the antimicrobial activities of phagocytic cells, mainly macrophages and neutrophils. The resident phagocytes in the lungs are the alveolar macrophages. They are in close proximity to the alveoli and responsible for the removal of incoming pathogens [143]. Phagocytosis of pathogens by alveolar macrophages is mediated by a variety of receptors including Fc and complement receptors (which recognize C3b stabilized on the bacterial surface). Following binding to the surface, macrophages phagocytize both opsonized and un-opsonized pathogens, which are internalized and killed in a sequence of discrete stages [144]. Depletion of alveolar macrophages in mice during pneumococcal pneumonia resulted in an increased lethality, indicating a major role of these phagocytic cells in host immune response to *S. pneumoniae* [145]. However, the bacterial clearance was not affected by macrophage depletion. Furthermore, the fact that opsonization of *S. pneumoniae* is critical for phagocytosis by macrophages [146, 147] and that murine alveolar macrophages express only low levels of the receptors for complement C3b/iC3b (CR1/CR3/CR4) [148] indicates that the protective effect of macrophages during pneumococcal pneumonia may not be mediated by the capacity of these cells to phagocytize and kill the bacterium but most probably by the production of inflammatory cytokines. Thus, alveolar macrophages can release TNF-α and IL-1 to stimulate the production of neutrophil chemoattractants such as CXC chemokines including IL-8, KC (keratinocyte chemoattractant) and MIP-2 (macrophage inflammatory protein 2) by epithelial cells [149-151]. The recruitment of neutrophils from the circulating blood into infected airspaces is crucial for the clearance of invading pneumococci since depletion of neutrophils rendered mice more susceptible to *S. pneumoniae* infection [152]. One major function of neutrophils consists of phagocytosis after recognition of serum opsonins such as complement and immunoglobulins that are attached to invading pathogens. Phagocytized bacteria are killed rapidly by proteolytic enzymes, antimicrobial proteins, and reactive oxygen species [153]. Another potent mechanism to eliminate pathogens is the formation of neutrophil extracellular traps (NETs). NETs are comprised of neutrophil chromatin associated with granule proteins that disarm and kill bacteria in an extracellular manner [154]. However, pneumococci can escape extracellular killing by degrading the DNA backbone of NETs via the surface endonuclease EndA [155]. Although neutrophils are potent effectors of bacterial clearance, their recruitment contributes to inflammatory tissue damage [156]. In this regard,

alveolar macrophages have been implicated to clear apoptotic neutrophils thereby preventing the release of potentially toxic or immunogenic intracellular contents [145].

### 1.2.3. The role of dendritic cells

An additional immune cell population residing in the lungs are the dendritic cells (DCs) [157]. DCs were first described by Ralph Steinman and Zanvil Cohn in 1973 [158]. These cells are antigen-presenting cells (APCs), which have the unique ability to induce antigen-specific primary immune responses [159-162]. Newly generated DC progenitors from the bone marrow circulate in the bloodstream and home to non-lymphoid tissues, where they reside as sentinels for invading pathogens in an immature stage with high phagocytic capacity (Fig. 2).

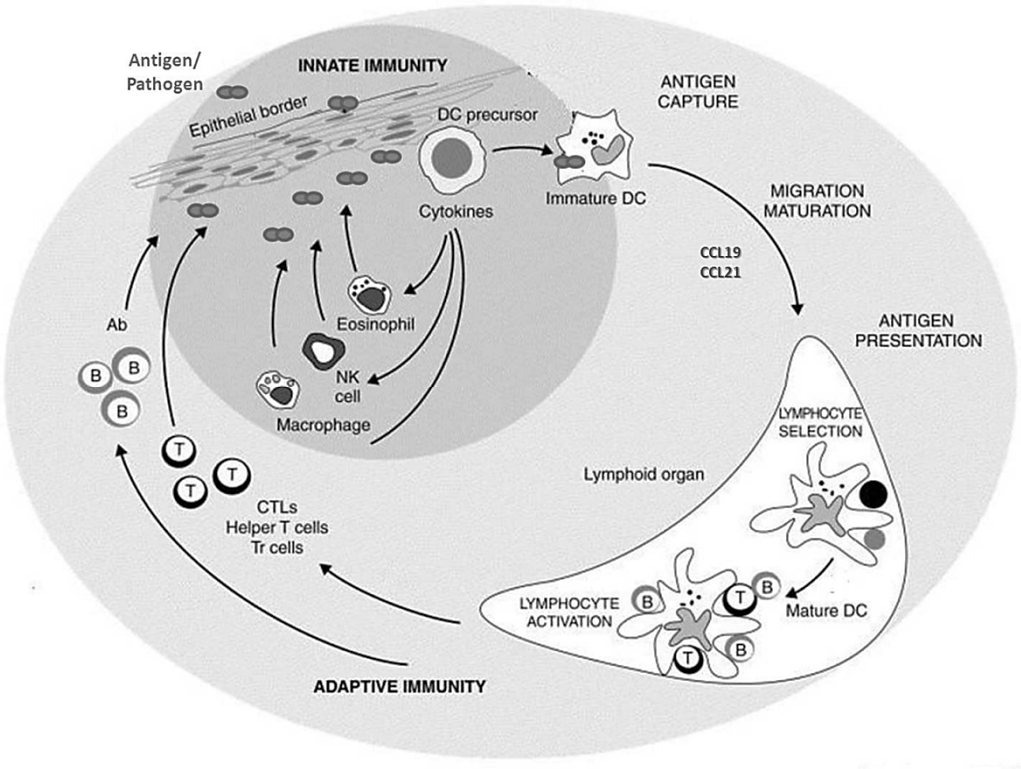


Figure 2: The life cycle of dendritic cells (DC). Circulating precursor DCs and immature DCs, situated in the tissue, can encounter antigens in the periphery. This leads to the secretion of cytokines such as interferon-alpha (IFN-α), which in turn can activate eosinophils, macrophages, and natural killer (NK) cells. After antigen capture, the DCs start a maturation program and migrate to lymphoid organs where they activate antigen-specific lymphocytes. After expansion and differentiation the activated T lymphocytes migrate to the infected tissue. T cells as well as DCs activate B cells to become plasma cells, which produce antibodies (Ab) that neutralize the pathogen. Adapted with modifications from Palucka and Banchereau, 2002 [163].

DCs can traverse the tight junctions of epithelial surfaces, interact directly with bacteria on the mucosal surface and control local inflammatory responses [164]. In the lungs DCs are in close proximity to alveolar epithelial and capillary endothelial cells and form a network of sentinel cells specialized to sample inhaled bacterial pathogens [165, 166]. Their encounter with antigens by phagocytosis, macropinocytosis, or endocytosis [167, 168] triggers their maturation and migration to secondary lymphoid organs, where they perform antigen-presentation functions. Migration of DCs involves a coordinated action of several chemokines. Upon maturation DCs down-regulate chemokine receptors which would drive their migration towards inflammatory sites (such as CCR1, CCR2 and CCR5) and concomitantly up-regulate a single known chemokine receptor, C-C chemokine receptor type 7 (CCR7) [169], and migrate directed by a gradient of the CCR7 ligands CCL19 and CCL21 to the draining lymph nodes [170, 171]. The maturation process is also associated with the up-regulation of the co-stimulatory molecules CD40, CD58, CD80, and CD86, change in morphology, and changes in the major histocompatibility complex class II (MHC class II) compartments [172]. Once in the lymph nodes DCs activate antigen-specific lymphocytes (Fig. 3). Besides activating naive T cells, DCs can also directly activate naive and memory B cells as well as enhance the differentiation of activated-naive B cells into plasma cells [172].

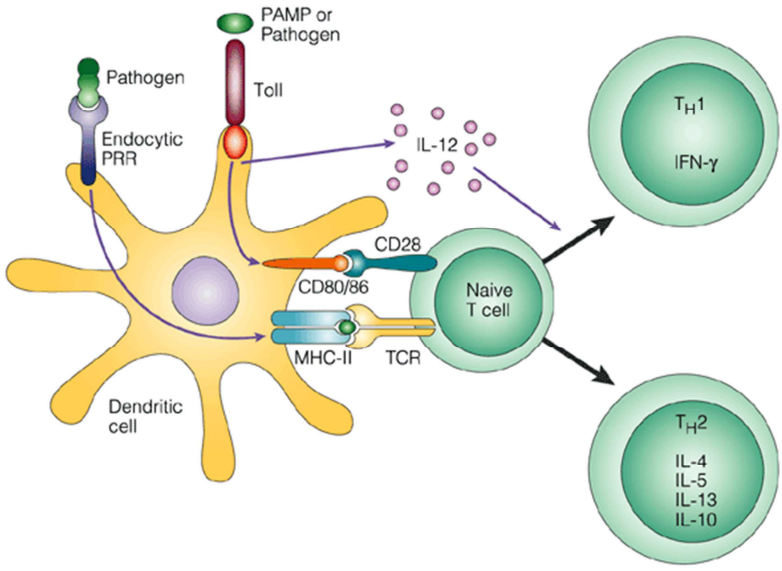


Figure 3: T cell activation and differentiation induced by DCs. DCs recognize pathogens via pathogen recognition receptors (PRRs) including Toll-like receptors and undergo a maturation process. Mature DCs express high levels of MHC and co-stimulatory molecules (CD80 and CD86), which are needed for efficient activation of naive T cells. TLRs do not only induce co-stimulatory molecules, they also contribute to the differentiation into T helper 1 ($T_H1$) effector cells by inducing expression of various cytokines, including IL-12. Adapted without modifications from Medzhitov, 2001 [129].

In addition to immature and mature differentiation stages, a third stage has been proposed by Lutz *et al*. [173] termed semi-mature or partially mature DCs. This semi-mature state is characterized by altered expression levels of surface molecules and a low or absent production of pro-inflammatory cytokines such as IL-6, TNF-α, IL-1ß and in particular IL-12 [173]. Such semi-mature DCs migrate to lymph nodes but induce tolerance in T cells rather than immunity. During infection, induction of semi-mature DCs results in delayed expression of immunity and enhanced pathogen survival. In this regard, there is evidence that different pathogen-derived components such as the filamentous hemagglutinin (FHA) and the adenylate cyclase toxin (CyaA) of *Bordetella pertussis* as well as the cholera toxin of *Vibrio cholerae* inhibit IL-12 production as well as the CD40 expression of DCs which results in the induction of regulatory T cells (Tregs) thereby providing a mechanism to escape protective immune responses [174-176]. Figure 4 shows schematically the different pathways of T cell activation.

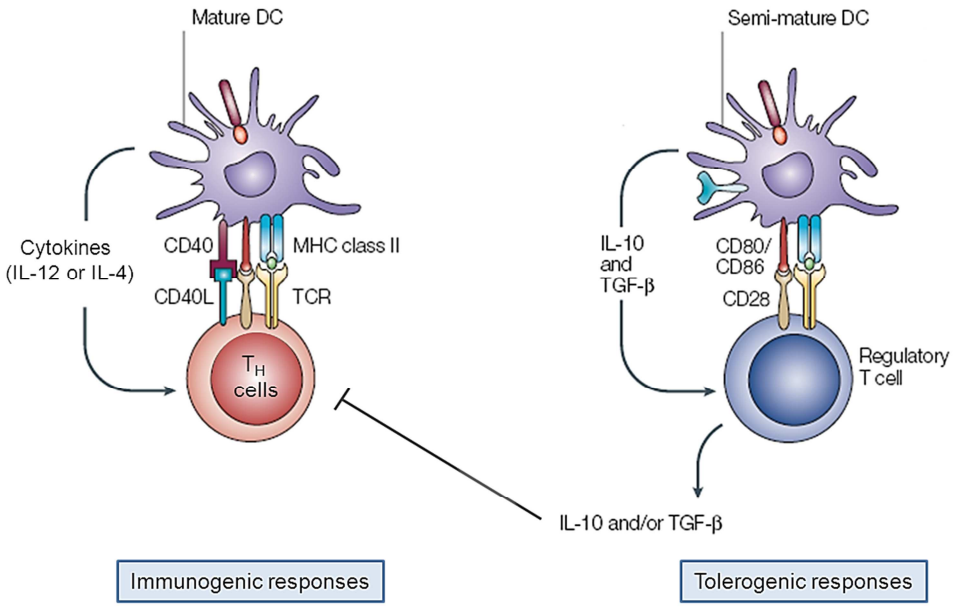


Figure 4: Schematic overview of the mechanisms leading to $T_H$ cell or Treg cell activation. Mature DCs upregulate cell-surface expression of CD40, CD80, CD86 and MHC class II as well as the production of cytokines such as IL-12 or IL-4, leading to $T_H$ cell induction. Some pathogen-derived molecules activate DCs to a semi-mature or intermediate phenotype by stimulation of IL-10 production and inhibition of IL-12 production of DCs, which promotes the induction of Treg cells. IL-10 and transforming growth factor-β (TGF-β) produced by Treg cells inhibit the activation of $T_H$ cells. Adapted with modifications from Mills, 2004 [177].

The trans-epithelial migration of DCs during normal physiological or inflammatory processes requires degradation of extracellular matrices (ECMs), which is largely mediated by the production of matrix metalloproteinases (MMPs). The MMP family consists of at least 24 endopeptidases with common motifs including propeptide and zinc-binding catalytic regions (Fig. 5) [178]. Collectively, MMPs are extracellular proteases that are capable of modifying almost all components of the extracellular matrix and the basal membrane including collagens, fibronectin, elastin, proteoglycans, and laminin [179, 180]. MMP-2 and MMP-9 (also referred to as gelatinase A and gelatinase B) most specifically cleave gelatin and type IV collagen, the major structural components of the ECM [96]. Human MMP-2 is secreted as a 72-kDa pro-form that is cleaved into a 64-kDa active form; the corresponding pro- and active forms of human MMP-9 have masses of 92 kDa and 83 kDa, respectively [181]. MMP-2 is known to be produced constitutively by tissue structural cells including fibroblasts and endothelial cells, whereas constitutive expression of MMP-9 is restricted to neutrophils [182] and eosinophils [183]. Inflammatory stimulation can lead to increased MMP-9 expression in many cell types including inflammatory cells such as macrophages [184], eosinophils [185], lymphocytes [186], natural killer cells [187], and DCs [188]. Apart from digesting components of the ECM, both MMP-2 and MMP-9 have certain chemokines and cytokines as their substrates. MMP-2 cleaves the pro-inflammatory molecule monocyte chemoattractant protein-3 (MCP-3) into the truncated anti-inflammatory form, which helps in dampening of inflammatory processes [189]. MMP-9 has more diverse effects: It degrades α1-antitrypsin, protecting neutrophil elastase activity [190], and potentiates the collagenolytic activity of MMP-13 [191] and fibroblasts in collagen gels [192]; it increases the chemotactic activity of CXCL8 (formerly human IL-8 or murine KC) for neutrophils 10-fold by cleavage of a six-amino acid peptide from CXCL8, but it also inactivates other neutrophil chemokines [193]. MMP-9 is also able to cleave the inactive membrane-bound forms of TNF-α and transforming growth factor-beta (TGF-ß) to generate their corresponding active forms [194, 195]. Similarly, both gelatinases are able to generate the active form of IL-1ß from its inactive pro-form [196]. Studies using mice deficient in MMP-9 expression have shown surprising results since these mice were protected against cerebral ischemia [197] and traumatic brain injury [198] compared to wild-type mice. These data implicate a pathophysiologic role for MMP-9, but the precise mechanisms involved remain to be fully elucidated.

The proteinases are highly regulated at different levels: 1) gene expression and protein secretion [199, 200], 2) activation of latent MMP pro-forms by different non-proteolytic agents and proteinases [199, 201], and 3) inhibition of activated MMPs. The most important

nonspecific inhibitor is α2-macroglobulin [202]. Specific inhibitors of MMPs are the tissue inhibitors of matrix metalloproteinases (TIMPs) [203]. TIMP-1 and TIMP-2 are capable of inhibiting MMPs by forming stoichiometric 1:1 complexes with active MMPs.

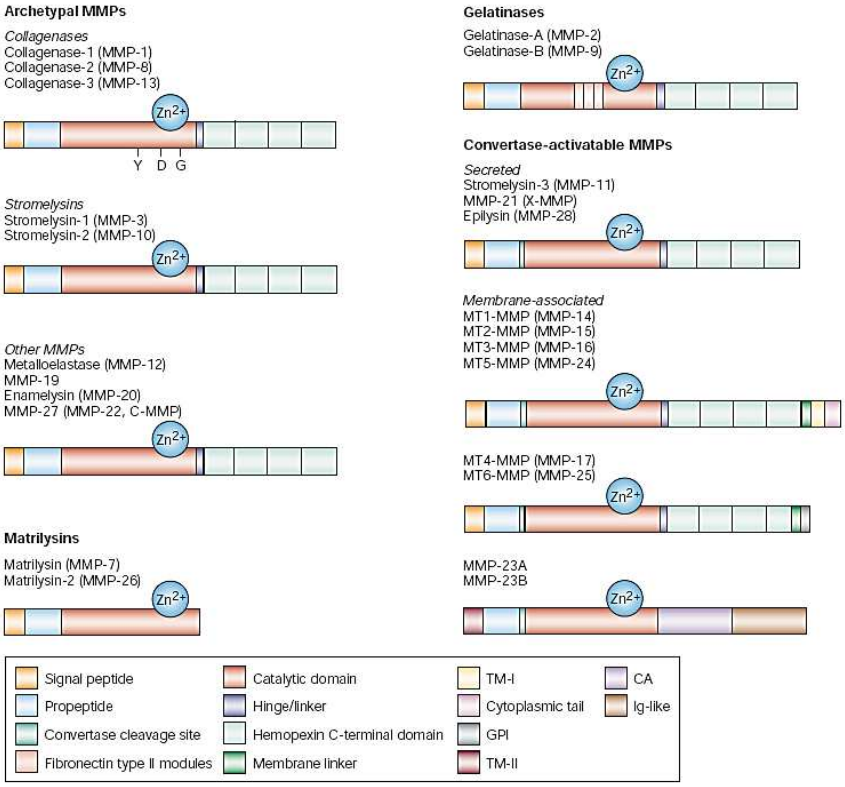


Figure 5: Classification of matrix metalloproteinases. On the basis of the domain organization the MMPs can be classified into four groups. Adapted without modifications from Overall and Otin, 2002 [178].

There is accumulating evidence supporting an important role for MMPs and TIMPs in a variety of pulmonary inflammatory disorders, including asthma [204], chronic obstructive pulmonary disease (COPD) [205, 206], acute lung injury [207], acute respiratory distress syndrome [208], and pulmonary fibrosis [209].

However, scarce information is available regarding the role played by DCs during *S. pneumoniae* infection. *In vitro* studies have shown that pneumococcal pneumolysin inhibits human DC maturation, induction of pro-inflammatory cytokines, and activation of the inflammasome [210]. In addition, Noske *et al.* [211] reported that expression of the pneumococcal adherence and virulence factor A (PavA) protected *S. pneumoniae* against recognition and phagocytosis by human DCs. Furthermore, cytokine production was delayed

and reduced when DCs were exposed to *S. pneumoniae* deficient in the expression of PavA [211]. The interactions between *S. pneumoniae* and DCs during *in vivo* infections has, however, not been investigated so far.

## 2. Aim of the study

*S. pneumoniae* is still a major cause of community-acquired infections of the respiratory tract, central nervous system and bloodstream. It is associated with significant morbidity and mortality, and poses a major economic burden to the healthcare system. The global emergence of antibiotic-resistant pneumococcal strains has complicated treatment decisions and increased the likelihood of treatment failure. More detailed understanding of the interactions between *S. pneumoniae* and the host may open up new avenues for the development of more effective treatment regimens, targeting not solely bacterial destruction, but also modulation of the host response.

Dendritic cells are potent antigen-presenting cells able to initiate and regulate innate and adaptive immune responses against invading pathogens. In the lung, dendritic cells are situated in immediate proximity to the respiratory epithelial cells, where they form an elaborate network that rapidly reacts to incoming pathogens. Despite the clinical relevance of pneumococcal pneumonia, scarce information is available regarding the role played by dendritic cells during this infection. Therefore, the aim of this study was to investigate the role of dendritic cells in the host response to *S. pneumoniae* lung infections.

# 3. Materials & Methods

## 3.1. Materials

### 3.1.1. Chemicals, solutions and kits

**Table 1: Chemicals, solutions, kits and their suppliers used in this study.**

| Chemical / Solution / Kit | Company |
|---|---|
| 2-Mercaptoethanol, 50 mM | Invitrogen, Karlsruhe, Germany |
| 3,8-diamino-5-ethyl-6-phenylphenantridiniumbromide (Ethidium Bromide) | Sigma Aldrich, Munich, Germany |
| Agarose NEEO Ultra quality | Carl Roth, Karlsruhe, Germany |
| Albumine, IgG free | Carl Roth, Karlsruhe, Germany |
| Ammonium chloride ($NH_4Cl$) | Merck, Darmstadt, Germany |
| Aprotinin | Sigma Aldrich, Munich, Germany |
| Bacto™ yeast extract | BD Pharmingen™, Heidelberg, Germany |
| BD Columbia Agar with 5% Sheep Blood | BD Pharmingen™, Heidelberg, Germany |
| Brij-35 (30% v/v) | Merck, Darmstadt, Germany |
| Bromophenol blue | Sigma Aldrich, Munich, Germany |
| Carrageenan type IVλ | Sigma Aldrich, Munich, Germany |
| Collagenase Type F | Sigma Aldrich, Munich, Germany |
| Coomassie Brilliant Blue G-250 | Serva Electrophoresis GmbH, Heidelberg, Germany |
| Deoxyribonuclease I (DNase I) | Qiagen, Hilden, Germany |
| Dimethyl sulfoxide (DMSO) | Sigma Aldrich, Munich, Germany |
| Diphtheria Toxin (DT) | Sigma Aldrich, Munich, Germany |
| Disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) | AppliChem, Darmstadt, Germany |
| Erythromycin | Sigma Aldrich, Munich, Germany |
| Ethanol, 96% (v/v) | J.T. Baker, Deventer, Netherlands |
| Ethylenediaminetetraacetic acid (EDTA) | AppliChem, Darmstadt, Germany |
| Evans Blue | Sigma Aldrich, Munich, Germany |
| Fetal calf serum (FCS) | Biochrom, Berlin, Germany |
| Gelatin (bovine) | Sigma Aldrich, Munich, Germany |

| | |
|---|---|
| GeneRuler™ DNA ladder mix, 100-10,000 bp | Fermentas, St. Leon-Rot, Germany |
| Gentamicin | Sigma Aldrich, Munich, Germany |
| Glycerine 86%, p.a. | Carl Roth, Karlsruhe, Germany |
| Hydrochloric acid (HCl), 37% | Carl Roth, Karlsruhe, Germany |
| Kanamycin | Carl Roth, Karlsruhe, Germany |
| Leupeptide | Sigma Aldrich, Munich, Germany |
| L-Glutamine (200 mM) | PAA Laboratories, Pasching, Austria |
| Lipopolysaccharide (LPS) of *E.coli* | Sigma Aldrich, Munich, Germany |
| Maxima™ SYBR Green qPCR Master Mix (2 ×) | Thermo Scientific, Bonn, Germany |
| M-MLV Reverse Transcriptase Kit | Promega, Mannheim, Germany |
| Nuclease-free water | Applied Biosystems/Ambion, TX, USA |
| OptiPrep™ density gradient | Axis-Shield, Heidelberg, Germany |
| Penicillin/Streptomycin, 100 × | PAA Laboratories, Pasching, Austria |
| Phenylmethyl sulfonyl fluoride (PMSF) | AppliChem, Darmstadt, Germany |
| Polyoxyethylene Sorbitan Monolaurate (Tween-20) | Serva Electrophoresis GmbH, Heidelberg, Germany |
| Potassium chloride (KCl) | Carl Roth, Karlsruhe, Germany |
| RNeasy Mini Kit | Qiagen, Hilden, Germany |
| RNeasy Midi Kit | Qiagen, Hilden, Germany |
| RPMI 1640 | Invitrogen, Karlsruhe, Germany |
| RevertAid First Strand cDNA synthesis Kit | Thermo Scientific, Bonn, Germany |
| Sodium carbonate ($Na_2CO_3$) | Merck, Darmstadt, Germany |
| Sodium chloride (NaCl) | Carl Roth, Karlsruhe, Germany |
| Sodium hydroxide (NaOH) | Carl Roth, Karlsruhe, Germany |
| Sodium dodecyl sulfate (SDS) | Sigma Aldrich, Munich, Germany |
| Sulfuric acid ($H_2SO_4$) | Carl Roth, Karlsruhe, Germany |
| Todd-Hewitt Broth | Sigma Aldrich, Munich, Germany |
| Triton X-100 | Carl Roth, Karlsruhe, Germany |
| Trizma® Base, ≥99.9% | Sigma Aldrich, Munich, Germany |
| Tryphan blue (0.4%) | Sigma Aldrich, Munich, Germany |

### 3.1.2. Antibodies

**Table 2: Antibodies and their suppliers used in this study.**

| Antibody | Company |
|---|---|
| Anti-mouse CD4-FITC | Invitrogen, Darmstadt, Germany |
| Anti-mouse CD8a-PE | Invitrogen, Darmstadt, Germany |
| Anti-mouse CD11b-APC | Invitrogen, Darmstadt, Germany |
| Anti-mouse CD11c-PE | Invitrogen, Darmstadt, Germany |
| Anti-mouse CD40-FITC | Invitrogen, Darmstadt, Germany |
| Anti-mouse CD80-FITC | Invitrogen, Darmstadt, Germany |
| Anti-mouse CD86-FITC | Invitrogen, Darmstadt, Germany |
| Anti-mouse CCR7-APC | Invitrogen, Darmstadt, Germany |
| Anti-mouse MHC class II-PE | Invitrogen, Darmstadt, Germany |
| Anti-mouse Ly6G-PE | Invitrogen, Darmstadt, Germany |
| BD OptEIA™ Mouse ELISA Kit (IL-6, IL-12, TNF-α) | BD Pharmingen™, Heidelberg, Germany |
| BD PE annexin V apoptosis detection kit I | BD Pharmingen™, Heidelberg, Germany |

### 3.1.3. Buffers & media

**Table 3: Composition and preparation of buffers and media.**

| Buffer / Medium | Amount of chemical / solution |
|---|---|
| 0.05% Tween/PBS (5 L) | 250 mL 20 × PBS, pH 7.4<br>2.5 mL Tween® 20<br>*ad* 5 L *Aq. dest*. |
| 0.5 µg/mL Ethidium Bromide (1 L) | 800 µl Ethidium Bromide<br>*ad* 1 L *Aq. dest*. |
| 0.5 M EDTA, pH 8.0 (1 L) | 186.12 g EDTA $Na_2 \cdot 2H_2O$<br>*ad* 1 L *Aq. dest*.<br>adjust pH 8 with NaOH |
| 1 M HCl (100 mL) | 8.4 mL 37% HCl<br>*ad* 100 mL *Aq. dest*. |
| 1 M NaOH (100 mL) | 4 g NaOH<br>*ad* 100 mL *Aq. dest*. |

| | |
|---|---|
| 1 M Tris, pH 8.8 (1 L) | 121.1 g Trizma Base<br>*ad* 1 L *Aq. dest.*<br>adjust pH 8.8 with HCl |
| 1% agarose gel (100 mL) | 1 g agarose<br>100 mL 1 M Tris, pH 8.8<br>Boil in microwave until agarose is melted |
| 100 mM $Na_2CO_3$, pH 9.6 (1L) | 10.6 g $Na_2CO_3$<br>*ad* 1 L *Aq. dest.*<br>adjust pH 9.6 with HCl |
| 1 × PBS, pH 7.4 (1 L) | 50 mL 20 × PBS, pH 7.4<br>*ad* 1 L *Aq. dest.*, autoclave |
| 1 × TAE, pH 8.5 (1L) | 20 mL 50 × TAE<br>*ad* 1 L *Aq. Dest.* |
| 20 × PBS, pH 7.4 (1 L) | 160 g NaCl<br>4 g KCl<br>15.2 g $Na_2HPO_4 \cdot 2H_2O$<br>4 g $KH_2PO_4$<br>*ad* 1 L *Aq. dest.*<br>adjust pH 7.4 with NaOH |
| 50 × TAE, pH 8.5 (1 L) | 242 g Trizma Base<br>57.1 mL Acetic Acid<br>100 mL of 0.5 M EDTA, pH 8.0<br>*ad* 1 L *Aq. dest.* |
| Ammonium-Chloride-Potassium buffer (ACK buffer) (1 L) | 1.0 g $KHCO_3$<br>8.29 g $NH_4Cl$<br>37.22 mg EDTA<br>$Na_2 \cdot 2H_2O$<br>*ad* 1 L *Aq. dest.*<br>adjust pH 7.4 with HCl<br>filter-sterilize (ø 0.22 µm),<br>store at room temperature (RT) |

| | |
|---|---|
| Ketamin-Rompun (anesthetic) | 1 mL 10% Ketamin<br>8.5 mL 0.9% NaCl<br>0.5 mL 2% Rompun<br>Inject 0.1 mL/10g body weight i.p. |
| Lysis buffer, pH 7.4 (1 L) | 11.69 g NaCl<br>1.86 g EDTA $Na_2 \cdot 2H_2O$<br>1.211g Trizma Base<br>100 mL glycerol<br>10 mL 0.1 M PMSF<br>1 mg leupeptide<br>28 mg aprotinin<br>*ad* 1 L *Aq. dest.*<br>adjust pH 7.4 with NaOH, store at -20°C |
| RPMI complete medium (500 mL) | 50 mL FCS<br>5 mL Penicillin/Streptomycin (1%)<br>5 mL 200 mM L-Glutamine |
| THY-Medium (1 L) | 30 g Todd-Hewitt-Broth<br>10 g Yeast extract<br>*ad* 1 L *Aq. dest.* and autoclave |
| Zymography separating gel (10%)<br>(adequate for 2 × 75mm gels) | 3 mL $ddH_2O$<br>2.36 mL 1.5M Tris, pH 8.8<br>90 µL 10% SDS<br>3 mL 30% Acryl/bis-acryl<br>600 µL gelatin (15mg/mL)<br>4.4 µL TEMED<br>44 µL APS (100mg/mL) |
| Zymography stacking gel (4%)<br>(adequate for 2 × 75mm gels) | 3.71 mL $ddH_2O$<br>550 µL 0.5M Tris, pH 6.8<br>50 µL 10% SDS<br>675 µL 30% Acryl/bis-acryl<br>7.5 µL TEMED<br>30 µL APS (100mg/mL) |

| | |
|---|---|
| Zymogrography 2 × SDS Sample Buffer (8 mL) | 1.0 mL 0.5 M Tris-HCl, pH 6.8<br>0.8 mL glycerol<br>1.6 mL 10% (w/v) SDS<br>0.2 mL 0.5% (w/v) bromophenol blue<br>*ad* 8 mL *Aq. dest.* |
| Zymography 4 × Tank Buffer (1 L) | 15.0 g Trisma Base<br>72.0 g glycine<br>5.0 g SDS<br>*ad* 1 L *Aq. dest.*<br>Store at 4 ºC, warm to 37 ºC before use if a precipitate forms. Use 1 × Tank buffer for electrophoresis. |
| Zymography 10 × LSCB (1 L) | 60.55 g Trisma Base (pH 7.6)<br>116.88 g NaCl<br>5.54 g $CaCl_2$<br>0.2% (w/v) Brij-35<br>*ad* 1 L *Aq. dest.* |
| Zymography Coomassie Brilliant Blue G-250 (1 L) | 62.5 mL 1.0% stock Coomassie G-250<br>250 mL methanol<br>50 mL 10% acetic acid<br>20 mM EDTA<br>*ad* 1 L *Aq. dest.* |

### 3.1.4. Materials & Instruments

**Table 4: Materials, instruments and their manufacturer used in this study.**

| | Instrument | Company |
|---|---|---|
| **Centrifugation** | Centrifuge 5804R | Eppendorf, Hamburg, Germany |
| | Centrifuge 5417R | Eppendorf, Hamburg, Germany |
| **Photometry** | BD™ LSRII Analyzer | BD Biosciences, CA, USA |
| | FACSCalibur™ flow cytometer | BD Biosciences, CA, USA |
| | Kodak Image Station 2000R | Kodak, NY, USA |
| | LightCycler® 480 Real-Time PCR System | Roche Diagnostics Deutschland GmbH, Mannheim, Germany |
| | Multitron Standard | Infors HT, Einsbach, Germany |
| | NanoDrop® 2000c | Thermo Fisher Scientific, IL, USA |
| | NOVASPEC II | Pharmacia LKB, Uppsala, Sweden |
| | Sunrise™ | Tecan Group Ltd., Männedorf, Switzerland |
| | 96-Well ELISA Microtiter plates, U bottom, clear | Greiner Bio-One Ltd, Stonehouse, United Kingdom |
| **Medical device** | BD Microlance™ 25G | BD Biosciences, CA, USA |
| | BD Microlance™ 26G 1/2 | BD Biosciences, CA, USA |
| | Inject® Solo (5 mL syringe) | Braun, Melsungen, Germany |
| | Omican®-F (1 mL syringe int. needle) | Braun, Melsungen, Germany |
| | Omnifix®-F Solo (1 mL syringe) | Braun, Melsungen, Germany |
| **Cell culture** | BD Falcon™ cell strainer (ø 100 µm) | BD Biosciences, CA, USA |
| | BX51 microscope | Olympus, Hamburg, Germany |
| | CellTrics® (ø 30 µm) | Partec, Münster, Germany |
| | GFL 1003 water bath | Gesellschaft für Labortechnik mbH, Burgwedel, Germany |
| | HERAcell 150i $CO_2$ incubator | Thermo Fisher Scientific, IL, USA |
| | Hemacytometer (Neubauer) | Marienfeld GmbH, Marienfeld, Germany |
| | TPP 96-well tissue culture plate | Sigma Aldrich, Munich, Germany |

| | | |
|---|---|---|
| | Wallac MicroBeta® TriLux | Perkin Elmer/ Wallac, Freiburg, Germany |
| | 96-Well ELISA Microtiter plates, F bottom, clear | Greiner Bio-One Ltd, Stonehouse, United Kingdom |
| **Other** | BRL Horizon®58 electrophoresis chamber | Invitrogen, Karlsruhe, Germany |
| | Dispersing aggregate PT-DA 2112/2 WEC | KINEMATICA AG, Lucerne, Switzerland |
| | Electrophoresis power supply ST606T | Invitrogen, Karlsruhe, Germany |
| | Eppendorf Research® PhysioCare Concept pipettes | Eppendorf, Hamburg, Germany |
| | Immuno 96 MicroWell™ Solid Plates | Thermo Fisher Scientific, IL, USA |
| | Millex® syringe filter units (ø 0.22 µm) | Sigma Aldrich, Munich, Germany |
| | MS2 minishaker | IKA, Staufen, Germany |
| | POLYTRON PT 2100 homogenizer | KINEMATICA AG, Lucerne, Switzerland |
| | Thermostat™ 5320 | Eppendorf, Hamburg, Germany |
| | Tpersonal Thermocycler | Biometra, Göttingen, Germany |

## 3.2. Methods

### 3.2.1. Bacterial strains and growth conditions

The *S. pneumoniae* strains D39 capsular serotype 2 [212] and *S. pneumoniae* serotype 4, strain TIGR4 [213] were grown at 37°C in Todd-Hewitt broth supplemented with 1% (w/v) yeast extract (THY-medium) and 10% of heat-inactivated fetal calf serum (FCS). The pneumolysin-deficient mutant strain D39Δ*ply* [56] was grown in the presence of 12.5 µg/mL erythromycin. Bacteria were grown to the mid-log phase ($OD_{600} \approx 0.4$), collected by centrifugation for 10 minutes at 4000 rpm, and washed twice with sterile PBS. For preparation of the inoculum, the bacterial suspension was diluted with PBS to the required

concentration and the number of viable cells determined after serial dilution and plating onto blood agar.

### 3.2.2. Mouse strains

BALB/c mice were purchased from Harlan-Winkelmann (Borchen, Germany), B6.FVB-Tg [Itgax-DTR/GFP] 57Lan/J (referred to as CD11c-DTR) transgenic mice [214] were obtained from Steffen Jung (The Weizmann Institute of Science, Rehovot, Israel) and backcrossed against the BALB/c background. These transgenic mice express a simian diphtheria toxin (DT) receptor under the control of the murine CD11c promoter region. Since the simian receptor is $10^3$-$10^5$ times more susceptible to DT than the murine DT-receptor (DTR) [215] this allows selective depletion of DCs following the administration of DT because all murine DC subsets express CD11c on the surface (Fig. 6).

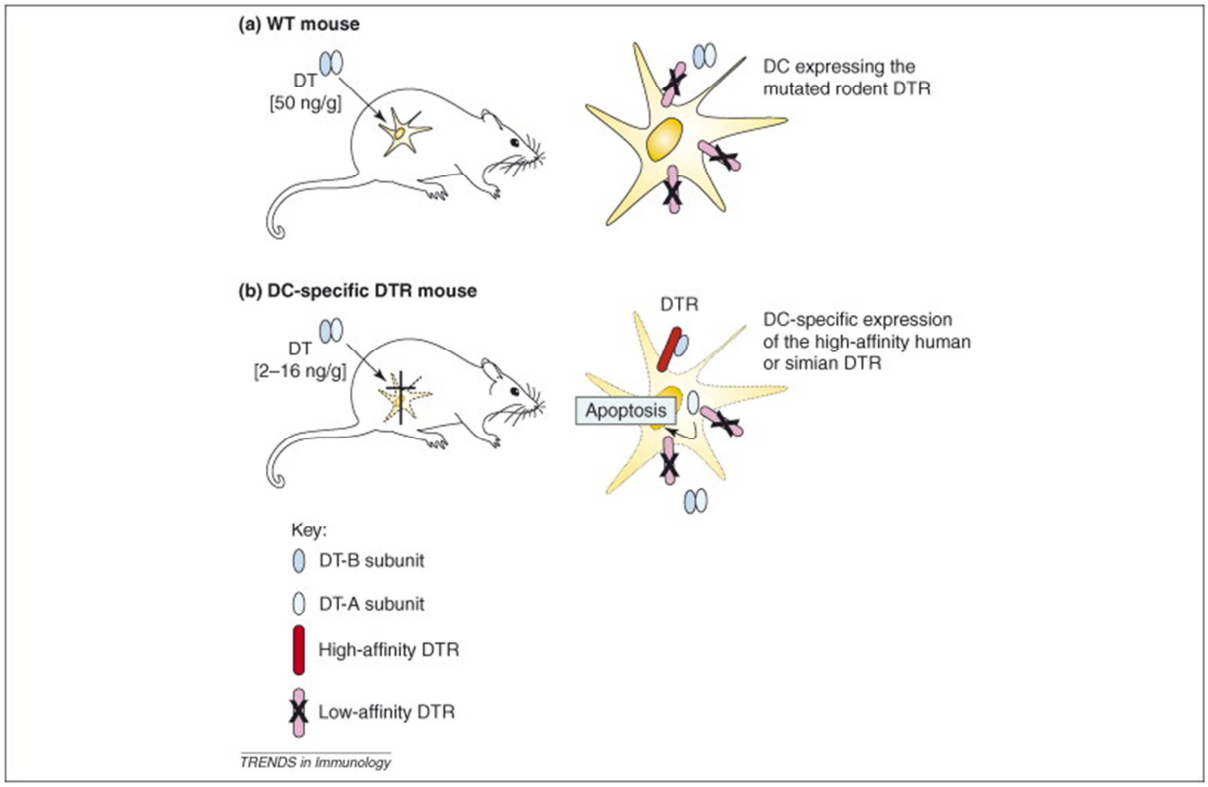


Figure 6: Depletion of DCs in CD11c-DTR mice. DCs of wild-type (wt) mice express the murine DTR with a low affinity to DT, whereas DCs of the transgenic CD11c-DTR mice express the high-affinity simian DTR. DT binds specifically to the simian DTR via the B subunit, that facilitates entry of the A subunit into the cell. The A subunit of DT inactivates elongation factor 2 through ADP ribosylation, thereby preventing protein synthesis and causing cell death. Adapted without modifications from Bennett and Clausen, 2007 [215].

Since one dose of DT is not enough to deplete DCs in the lungs and repeated administration has been shown to be lethal to CD11c-DTR mice, bone marrow chimera mice were generated as previously described [216]. Briefly, $2.5 \times 10^5$ bone marrow cells from CD11c-DTR donor mice were transferred intravenously into BALB/c mice, which were lethally irradiated (950 rads) 24 h earlier. The chimeric animals (referred to as CD11c-DTR chimera mice) were housed in individually ventilated pathogen-free cages and treated for 1 week with 0.04% Baytril together with 0.054% glucose in their drinking water. The CD11c-DTR chimeras were ready for experimental use 8 weeks after reconstitution. For continuous depletion of DCs, CD11c-DTR chimera mice received a daily intraperitoneal injection of DT (8 ng/g body weight in 200 µL PBS) starting two days before infection. DT treatment effectively depleted DCs from the lungs of CD11c-DTR chimera mice for up to 4 days (Fig. 7).

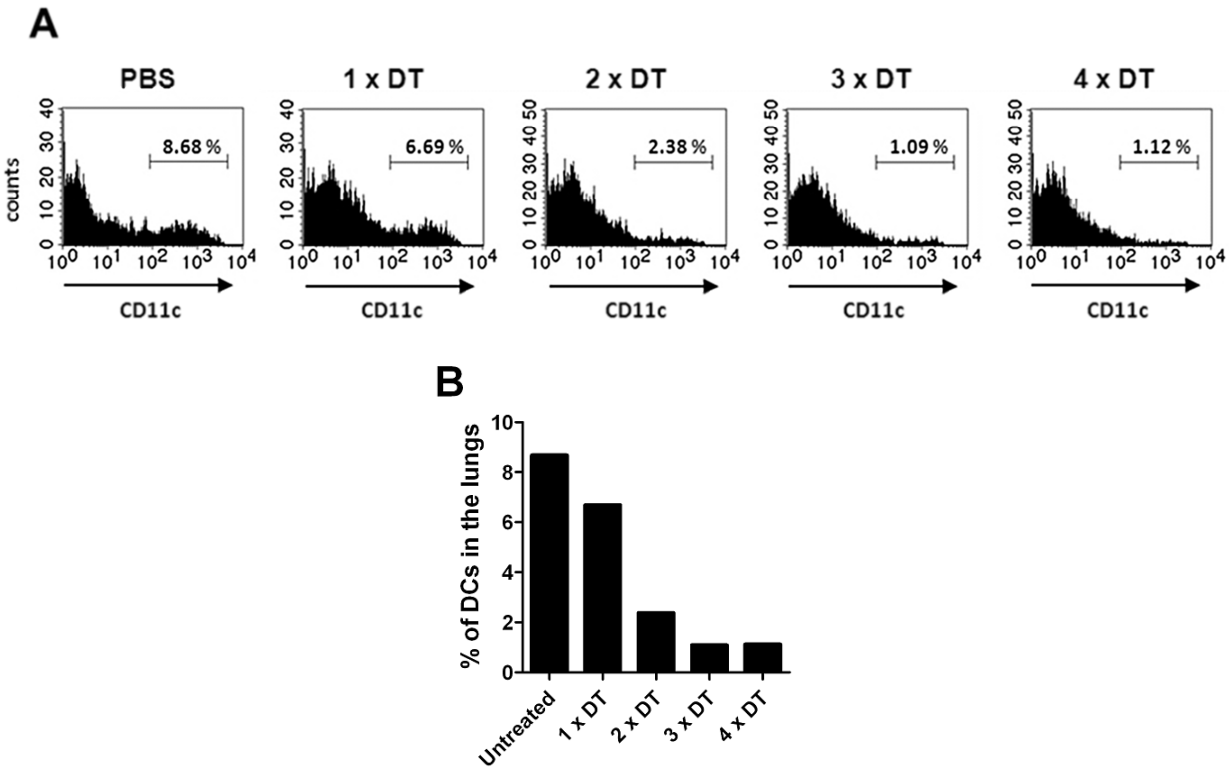


Figure 7: DC depletion efficiency in the lungs of CD11c-DTR chimera mice after treatment with consecutive doses of DT. CD11c-DTR chimera mice were injected with 1, 2, 3 or 4 successive doses of DT (8 ng/g body weight) or PBS. Lungs were digested and transformed into single cell suspensions 24 h after treatment and stained with anti-mouse CD11c antibodies for flow cytometry analysis. (A) Representative histogram of CD11c expression on lung cells of PBS-treated or DT-treated CD11c-DTR chimera mice. (B) Percentage of $CD11c^+$ cells in the lungs of PBS-treated or DT-treated CD11c-DTR chimera mice.

### 3.2.3. Double depletion of DCs and neutrophils

CD11c-DTR chimera mice were injected intraperitoneally daily with DT (8 ng/g body weight in 200 µL PBS) or with 200 µL of sterile PBS starting two days before infection and injected intravenously with 100 µg of anti-mouse RB6 antibody one day before bacterial inoculation. Control mice received equivalent amounts of isotype control antibodies in sterile PBS. The efficiency of neutrophil depletion was verified by flow cytometric analysis of peripheral blood obtained by retro-orbital bleeding of mice prior to and two days after anti-mouse RB6 antibody or mock treatment (Fig. 8). Briefly, blood was taken with a microhematocrit capillary tube and collected in a tube containing 300 µL with 2 U/mL heparin in 1 × PBS. The blood was centrifuged at 600 × g for 3 min at room temperature (RT) and the supernatant was discarded. While vortexing the cells, 9 mL of deionised water was added followed by 3 mL of 4 × PBS to lyse the erythrocytes. The cell suspension was further centrifuged, the pellet resuspended in 1 × PBS and the cells stained with PE-conjugated anti-mouse Ly6G antibodies for flow cytometry analysis.

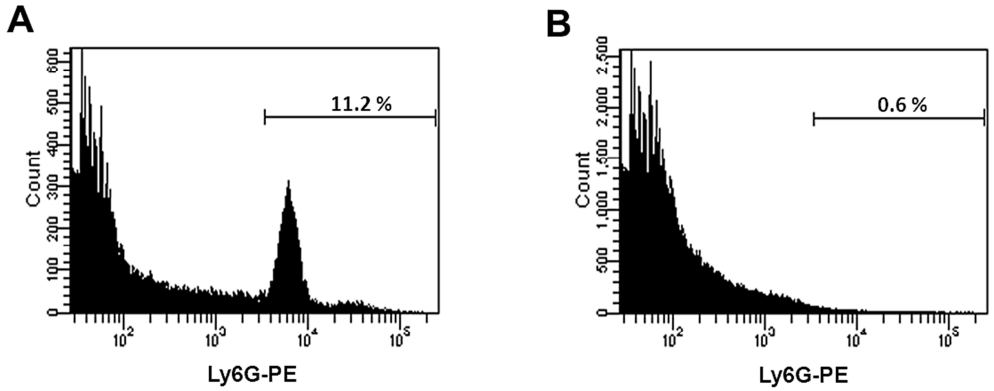


Figure 8: Neutrophil depletion efficiency in CD11c-DTR chimera mice after anti-mouse RB6 treatment. CD11c-DTR chimeras were depleted of neutrophils by intravenous injection of 100 µg purified anti-mouse RB6. Neutrophils of the blood were stained with PE-conjugated anti-mouse Ly6G for flow cytometry analysis. Representative histograms of Ly6G-positive neutrophils in the blood of a CD11c-DTR chimera mouse before (A) and 48 h after treatment with anti-mouse RB6 antibodies (B).

### 3.2.4. Infection model of pneumococcal pneumonia

For intranasal infection with *S. pneumoniae*, mice were anesthetized by intraperitoneal injection with ketamine-rompun solution (see Tab. 3) and inoculated intranasally with $1 \times 10^8$ CFU (colony forming units) of *S. pneumoniae*. At specified time points after bacterial inoculation, mice were euthanized by $CO_2$ asphyxiation, the organs were aseptically collected

and bacterial burdens were determined by preparing homogenates in 5 mL PBS and plating 10-fold serial dilutions on blood agar. Colonies were counted after overnight incubation at 37°C. For collection of serum, blood was collected at time of sacrifice, centrifuged at 5000 rpm for 10 min, and frozen at -80°C until use for further analysis.

All animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). All experiments were approved by the ethical board Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg in Germany (Permit No. 33.11.42502-04-118/08).

### 3.2.5. Detection of cytokines and chemokines

Cytokines/chemokines were analyzed either by ELISA (enzyme-linked immunosorbent assay) according to the recommendations of the manufacturer using matched antibody pairs and recombinant cytokines as standards or by multiplex bead technology according to the manufacturer's protocol. Briefly, for ELISA 96-well microtiter plates were coated with the purified anti-mouse capture antibody targeting the respective cytokine and incubated overnight at 4°C. The wells were washed with 0.05% Tween/PBS and then blocked with 200 µL of 10% FCS in PBS for 1 h at RT before samples and the appropriate standard (100 µL/well) were added. After incubation for 2 h at RT, the wells were washed again and the matching biotinylated anti-mouse detection antibody was applied. Detection was carried out with Streptavidin-horseradish peroxidase conjugated secondary antibody and the plates developed using TMB (100 µL/well) as substrate. The reaction was stopped by the addition of 2N $H_2SO_4$ solution (50 µL/well). The absorbance was read at 450 nm versus 570 nm using the Sunrise absorbance reader and Magellan$^{TM}$3 software. The background signal obtained from wells incubated with only assay buffer was subtracted before the cytokine concentrations were calculated based on the standard curve generated using recombinant cytokines. Samples and standards were at least measured in duplicates.

For determination of cytokine levels in lung tissue, lungs were homogenized in 1.5 mL lysis buffer (see Tab. 3) with a tissue grinder. Homogenized lung supernatants were collected by centrifugation at 13000 rpm for 10 min at 4°C, aliquoted and stored at -80°C until use for further analysis.

### 3.2.6. Isolation of lung cells for flow cytometry analysis

For analysis of immune cells recruited into the lungs during infection, the lungs were shredded into small pieces and enzymatically digested with 1 mg/mL of collagenase F and 50 U of DNase I in 500 µL RPMI 1640 at 37°C and 5% $CO_2$ for 30 min. The reaction was stopped by adding 1 mL of 10 mM EDTA (see Tab. 3) followed by incubation for 10 min on ice. The resulting tissue/cell suspension was filtered through a 100-µm strainer, centrifuged at 900 rpm for 8 min and the red blood cells lysed by adding 3 mL of ACK buffer (see Tab. 3) to the pellet. The cell suspension was centrifuged for 10 min at 900 rpm, resuspended in 1 mL RPMI 1640, counted and stained for flow cytometry analysis.

Cells were incubated for 5 min with 0.5 µL purified rat anti-mouse CD32/CD16 antibodies to block the Fc receptors, followed by incubation either with FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD8a, PE-conjugated anti-mouse CD11c, APC-conjugated anti-mouse CD11b, PE-conjugated anti-mouse Ly6G alone or in combination. After incubation for 30 min at 4°C, cells were washed and flow cytometry analysis was performed using the BD™ LSR II flow cytometer.

### 3.2.7. Gentamicin protection assay

To determine the amount of intracellular *S. pneumoniae* within lymph node cells, mediastinal lymph nodes were taken at 24 and 48 h after bacterial inoculation from *S. pneumoniae*-infected BALB/c mice. A single cell suspension was generated by carefully grinding the lymph nodes through a nylon sieve (ø 100 µm) with the back of a plunger. The cells were centrifuged at 900 rpm for 8 min and the supernatant plated in 10-fold serial dilutions onto blood agar plates to determine the number of extracellular bacteria. The cells were incubated with 200 µL ACK buffer for 2 min at room temperature to lyse the erythrocytes. The procedure was stopped by adding 400 µL 1 × PBS and subsequent centrifugation at 900 rpm. To eliminate any extracellular bacteria, the cell pellet was treated with gentamicin (100 µg/mL) for 2 h and afterwards collected by centrifugation. The cell pellet was disrupted after treatment with 50 µL dd$H_2O$, and the amount of viable intracellular bacteria was determined after serial plating onto blood agar.

### 3.2.8. Generation of bone marrow-derived dendritic cells

BALB/c mice were sacrificed by $CO_2$ asphyxiation and bone marrow cells were flushed from both femurs and tibias. Progenitor cells were resuspended, adjusted to a concentration of 1 × $10^6$/mL and cultured for 6-8 days at 37°C and 5% $CO_2$ in RPMI 1640 with 5% FCS, 1% penicillin/streptomycin, 50 ng/mL of recombinant mouse GM-CSF (granulocyte-macrophage colony-stimulating factor), and 10 ng/mL IL-4 (interleukin 4). DCs were gently washed (900 rpm) and fed with fresh medium supplemented with GM-CSF and IL-4 on day 2 and 5. On day 7, the DC fraction was enriched using an OptiPrep™ gradient according to the manufacturer's instructions. The purity of the resulting cell population consisted of >80% of DCs as determined by flow cytometry analysis using PE-conjugated anti-mouse CD11c antibodies.

### 3.2.9. *In vitro* infection of bone marrow-derived DCs

Bone marrow-derived DCs were seeded in 48-well plates at a density of 5 × $10^6$/mL and infected with *S. pneumoniae* at a multiplicity of infection (MOI) of 20 bacteria per DC unless otherwise noted. Infected DCs were incubated for 90 min in antibiotic-free RPMI medium, washed twice with sterile 1 × PBS to remove unbound bacteria and further incubated in RPMI 1640 supplemented with 5% FCS, 100 µg/mL of gentamicin and 10 µg/mL of penicillin. Stimulation with 1 µg/mL lipopolysaccharide (LPS) of *Escherichia coli* was used as a positive control for flow cytometry analysis of maturation markers and cytokine production. DCs were collected by centrifugation 24 h after infection, washed with 1 × PBS and stained for flow cytometry analysis. Antibodies used for the flow cytometry analysis of maturation markers were: PE-conjugated anti-mouse CD11c, FITC-conjugated anti-mouse CD40, FITC-conjugated anti-mouse CD80, FITC-conjugated anti-mouse CD86, FITC-conjugated anti-mouse MHC-II and APC-conjugated anti-mouse CCR7. Culture supernatant was also collected and stored at -80°C for later determination of cytokine concentration and gelatin gel zymography.

### 3.2.10. DC viability test

Viability of DCs during *in vitro* studies was determined using the PE Annexin V apoptosis detection kit I. Therefore, bone marrow-derived DCs were harvested by centrifugation (900 rpm, 8 min), washed twice with cold 1 × PBS and resuspended in 1 × binding buffer at a

concentration of 1 × $10^6$/mL. 100 µL of the solution (containing 1 × $10^5$ cells) were transferred to a 5 mL tube and stained with 5 µL PE Annexin V and 5 µL 7-AAD. The cells were gently vortexed and incubated for 15 min at RT in the dark. After adding 400 µL of 1 × binding buffer the cells were analyzed by flow cytometry.

### 3.2.11. Analysis of gene expression

Real-time quantitative RT-PCR was performed to measure the mRNA expression levels of *mmp-9* gene. Total RNA was extracted from cultured DCs using an RNeasy Mini Kit and from lung tissue using an RNeasy Midi Kit according to the manufacturer's recommendations. RNA was reverse transcribed and cDNA synthesis performed using the RevertAid$^{TM}$ First Strand cDNA synthesis kit. PCR amplification was performed using the LightCycler 480 Real-Time PCR system with the Maxima SYBR Green qPCR Master Mix. RT-PCR was performed in a 20 µl-volume reaction containing 5 µL cDNA, 2 µL specific primer mix (10 pmol), and 10 µL 2 × conc. Mastermix (SYBR Green qPCR). Thermal cycling consisted of denaturation for 10 min at 95° C followed by 50 cycles of 30 sec at 95°C, 30 sec at 59°C and 45 sec at 72°C. The sequence for MMP-9 sense primer was: 5'-GGGAAGGCTCTGCTGTTCAGC-3', and for antisense primer: 5'-TCTAGAGACTTGCACTGCACG-3'. The sequence for ß-actin sense primer was: 5'-TGGAATCCTGTGGCATCCATGAAA-3' and antisense primer: 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'. Cycle threshold values for MMP-9 were normalized to the housekeeping gene *β-actin*. The data were calculated using the Pfaffl equation [217] and expressed as a ratio of the relative mRNA expression in infected samples to that in uninfected controls.

### 3.2.12. Gelatin gel zymography

Enzymatic activity of MMP-9 in culture supernatants of bone marrow-derived DCs was determined by SDS-PAGE gelatin gel zymography. Gelatinases like MMP-9 degrade the gelatin matrix in the gel, leaving a clear band after staining the gel for protein [218]. Culture supernatant of pneumococcal infected and uninfected bone marrow-derived DCs were taken 6 and 24 h after infection, the protein concentrations determined using the Bradford method [219], and the samples mixed with equal volumes of 2 × SDS sample buffer (see Tab. 3). The samples were electrophoresed in 10% SDS-PAGE containing 0.1% (w/v) bovine gelatin. Gels

were incubated with gentle agitation in renaturing buffer (2.5% Triton X-100) at RT for 20 min and then incubated for at least 72h at 37°C in 1 × LSCB buffer containing 5 mM $CaCl_2$, 0.2 M NaCl, 0.02% (w/v) Brij-35, and 50 mM Tris (pH 7.6). Thereafter, gels were stained 1 h with Coomassie Dye containing 0.06% Coomassie Brilliant Dye G, 25% (v/v) methanol, 0.005% acidic acid, and 20mM EDTA. After destaining with 10% acidic acid, gels were imaged on a Cano Scan 9000F.

### 3.2.13. *In vivo* vascular permeability assay

Bone marrow-derived DCs were cultured at a density of $5 \times 10^5$/mL in antibiotic-free RPMI 1640 and infected with *S. pneumoniae* at a MOI of 10 bacteria per DC for 90 min. Bone marrow-derived DCs were then washed and further incubated in the presence of antibiotics (100 µg/mL gentamicin, 10 µg/mL penicillin) at 37°C and 5% $CO_2$. The culture supernatant was collected after 24 h of culture and stored at -80°C until use. Medium from uninfected DCs as well as medium where only bacteria were added were used as control. Evans blue (30mg/kg body weight) was injected intravenously into BALB/c mice, and the mice were subsequently anesthetized and the dorsal skin carefully shaved. 50 µL of the supernatant was injected intradermally into the dorsal skin of the mice. 1 h later the mice were sacrificed, pictures of the injection site were taken from the basal site of the skin and the size of the Evans blue spots measured before they were resected. For quantification of the vascular leakage the Evans blue was extracted by incubation of the tissue in 500 µL of formamide at 60°C for 48 h and subsequently analyzed by spectrophotometry at 620 nm using the Sunrise absorbance reader and Magellan$^{TM}$3 software. For the generation of a standard Evans blue solution was diluted 10-fold in formamide.

### 3.2.14. Statistical analysis

All data were analyzed in Microsoft Excel 2007 or GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Unless otherwise specified, results are presented as the mean ± SD. Comparison between groups was performed by the use of a two-tailed t-test or one-way ANOVA. P values $\leq 0.05$ were considered as significant.

# 4. Results

## 4.1. Depletion of DCs results in enhanced resistance to *S. pneumoniae* infection

To determine the importance of DCs in host defense against *S. pneumoniae*, a CD11c-DTR transgenic mouse line that permits conditional transient depletion of DCs by administration of DT was used. In these mice, DCs can be specifically depleted since $CD11c^+$ cells (which are DCs and alveolar macrophages) express the simian DTR on their surface. This receptor is 1000 times more susceptible to DT than the murine receptor. Endocytosis of DT after binding via its B subunit to the simian DTR results in protease-mediated splitting of the A and B subunits. The subunit B forms pores in the endosome membrane, thereby facilitating the release of the A subunit into the cytoplasm of the cell. The A subunit catalyzes the ADP-ribosylation of elongation factor 2 (EF-2). Since EF-2 is needed for the moving of tRNA from the A-site to the P-site of the ribosome during protein translation, ADP-ribosylation of EF-2 inhibits protein synthesis and thereby causes cell death [220].

Generally, CD11c-DTR mice allow depletion of DCs only for two days, and do not survive a second diphtheria toxin injection [214]. To enable prolonged depletion of DCs by multiple DT treatments, bone marrow chimeras were generated by transferring bone marrow from CD11c-DTR mice into lethally irradiated BALB/c mice as previously described [221]. CD11c-DTR chimera mice were depleted of DCs and infected intranasally with a sublethal inoculum of *S. pneumoniae* strain D39 ($1 \times 10^8$ CFU). Bacterial loads were monitored in the lungs, mediastinal lymph nodes, blood and spleen at 24, 48 and 72 h after bacterial inoculation.

Depletion of DCs rendered CD11c-DTR chimera mice significantly more resistant to infection with *S. pneumoniae* than non-depleted animals. Thus, depletion of DCs significantly reduced the amount of *S. pneumoniae* recovered from the lungs of infected mice at 48 and 72 h of infection (Fig. 9A). *S. pneumoniae* disseminated from the lungs first to the mediastinal lymph nodes and then to the blood and the systemic organs as shown by the detection of pneumococci in the lymph nodes at 24 h of infection as well as in the blood and the spleen at 48 h after bacterial inoculation (Fig. 9B-9D). Furthermore, *S. pneumoniae* disseminated to the lymph nodes to a lesser extent in DC-depleted than in non-depleted CD11c-DTR chimera mice (Fig. 9B).

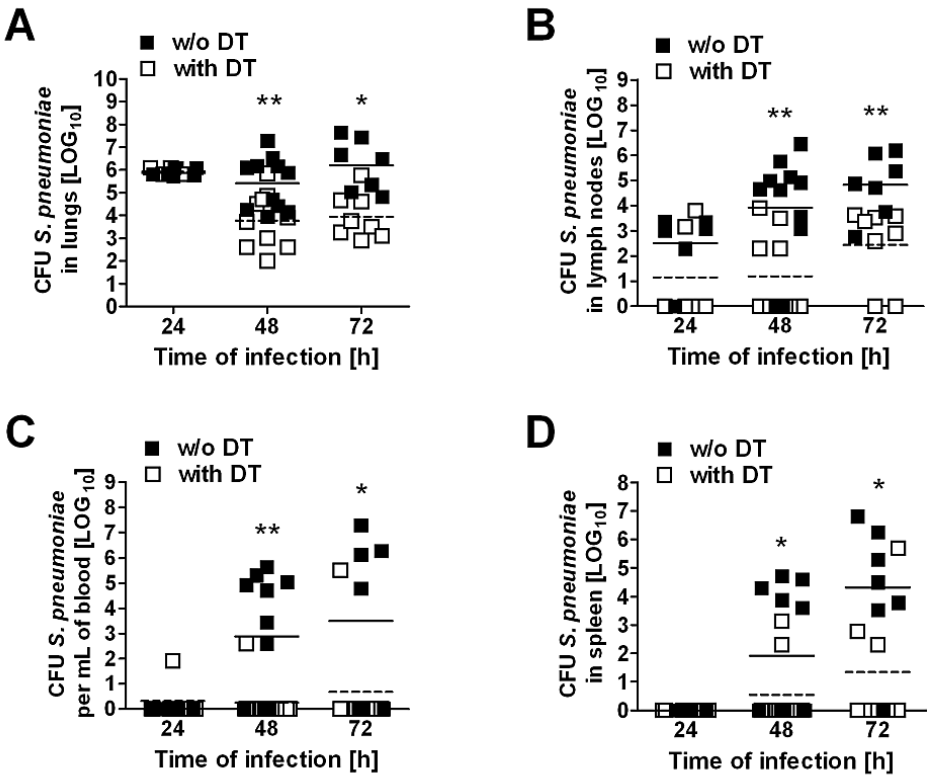


Figure 9: Bacterial burdens in the organs of DC-depleted and non-depleted mice after respiratory challenge with *S. pneumoniae*. DC-depleted (white symbols) and non-depleted (black symbols) CD11c-DTR chimera mice were intranasally inoculated with $1 \times 10^8$ CFU of *S. pneumoniae* D39 and the bacterial loads determined in the lungs (A), mediastinal lymph nodes (B), blood (C) and spleen (D) at progressive time points after bacterial inoculation. Each symbol represents an individual animal. Horizontal solid lines indicate the mean value of non-depleted mice, whereas broken lines designate the mean value of DC-depleted animals. One representative experiment out of three is shown. *, $p < 0.05$ and **, $p < 0.01$.

It has been reported that treatment of CD11c-DTR mice with DT can result in the depletion of alveolar macrophages in addition to DCs [222]. However, it has been reported that *in vivo* depletion of alveolar macrophages alone via intranasal instillation of liposomal dichloromethylene-bisphosphonate before infection with *S. pneumoniae* does not affect the bacterial burdens in the lungs or blood of infected mice although it significantly increased the mortality of infected animals [145]. Therefore, the benefit conferred by the depletion of $CD11c^+$ cells on the course of *S. pneumoniae* infection can be attributed to the depletion of DCs rather than the depletion of alveolar macrophages.

To determine if the beneficial effect of DC-depletion in CD11c-DTR chimera mice is strain specific, the DC-depleted and non-depleted mice were intranasally inoculated with the *S. pneumoniae* strain TIGR4. This strain is encapsulated and has been shown to be highly invasive and virulent in mice [213]. Inoculation of BALB/c mice with a dose of

*S. pneumoniae* strain TIGR4 similar to that used for infections with strain D39 ($1 \times 10^8$ CFU) resulted in enhanced mortality starting 24 h after bacterial inoculation (data not shown). These results demonstrated the superior virulence of *S. pneumoniae* TIGR4 compared to *S. pneumoniae* strain D39. Therefore, CD11c-DTR chimera mice were infected with a lower dose of *S. pneumoniae* TIGR4 ($1 \times 10^7$ CFU) which resulted in significantly reduced amounts of bacteria in the mediastinal lymph nodes, blood and spleen at 24 h of infection in DC-depleted mice compared to non-depleted animals (Fig. 10). Similar to infection with *S. pneumoniae* D39 there was no difference in the bacterial loads in the lungs at this time of infection. These results indicate that the increased resistance of DC-depleted CD11c-DTR chimera mice to pneumococcal pneumonia was not strain specific.

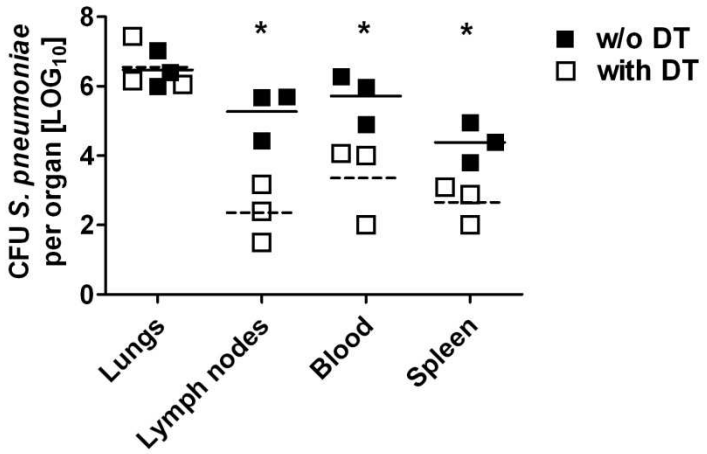


Figure 10: Bacterial burdens in the organs of DC-depleted and non-depleted mice after intranasal infection with *S. pneumoniae* strain TIGR4. DC-depleted (white symbols) and non-depleted (black symbols) CD11c-DTR chimera mice were intranasally inoculated with $1 \times 10^7$ CFU of *S. pneumoniae* TIGR4 and the bacterial loads were determined in the lungs, mediastinal lymph nodes, blood and spleen at 24 h post infection. Each symbol represents an individual animal. Solid horizontal lines indicate the mean value of non-depleted mice, whereas broken lines designate the mean value of DC-depleted animals. One representative experiment out of two is shown. *, $p < 0.05$.

A potential effect of DT *per se* on the course of pneumococcal infection was examined by administering DT (or PBS as a control) daily into normal BALB/c mice. The application of DT intraperitoneally started two days before infection with *S. pneumoniae* D39. DT-treated BALB/c mice exhibited no significant differences in the bacterial loads in the organs at 24 and 48 h of infection (Fig. 11). This result demonstrates that DT *per se* is not responsible for the enhanced resistance of DT-treated CD11c-CTR chimera mice to *S. pneumoniae* infection.

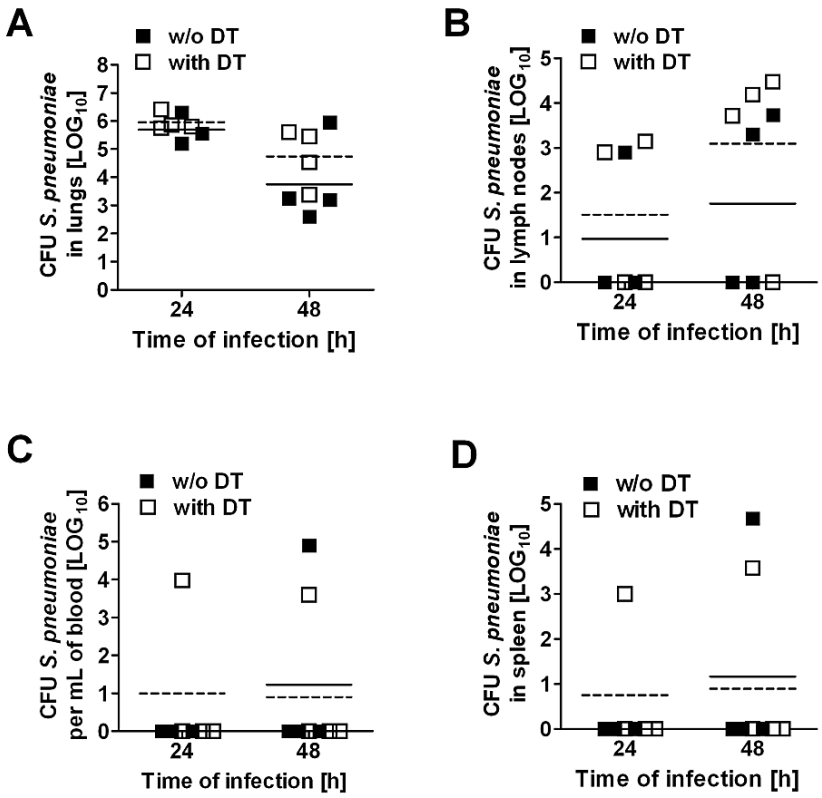


Figure 11: Bacterial burdens in the organs of BALB/c mice after intraperitoneal challenge with DT or PBS and subsequent intranasal infection with *S. pneumoniae*. DT-treated (white symbols) or PBS-treated (black symbols) BALB/c mice were intranasally inoculated with $1 \times 10^8$ CFU of *S. pneumoniae* D39 and the bacterial loads determined in the lungs (A), mediastinal lymph nodes (B), blood (C) and spleen (D) at 24 and 48 h of infection. Each symbol represents an individual animal. Horizontal solid lines indicate the mean value of PBS-treated mice, whereas broken lines designate the mean value of DT-treated animals. One representative experiment out of two is shown.

## 4.2. DC-depleted mice exhibit lower levels of systemic inflammation after intranasal inoculation with *S. pneumoniae* than non-depleted mice

Among the factors important for orchestrating the inflammatory responses are cytokines as well as chemokines because they are responsible for the recruitment of leukocytes to the site of infection. Determination of the serum levels of cytokines/chemokines in DC-depleted and non-depleted mice at progressive time points after bacterial inoculation demonstrated that the systemic inflammatory response triggered by *S. pneumoniae* was much more tempered in the absence of DCs. Thus, significantly lower levels of circulating serum cytokines IL-6 (Fig. 12A) and IFN-γ (Fig. 12B) as well as serum chemokines IP-10 (interferon gamma-induced protein 10) (Fig. 12C), MIG (monokine induced by gamma-interferon) (Fig. 12D) and KC (Fig. 12E) were detected at 72 h of infection in DC-depleted rather than non-depleted mice.

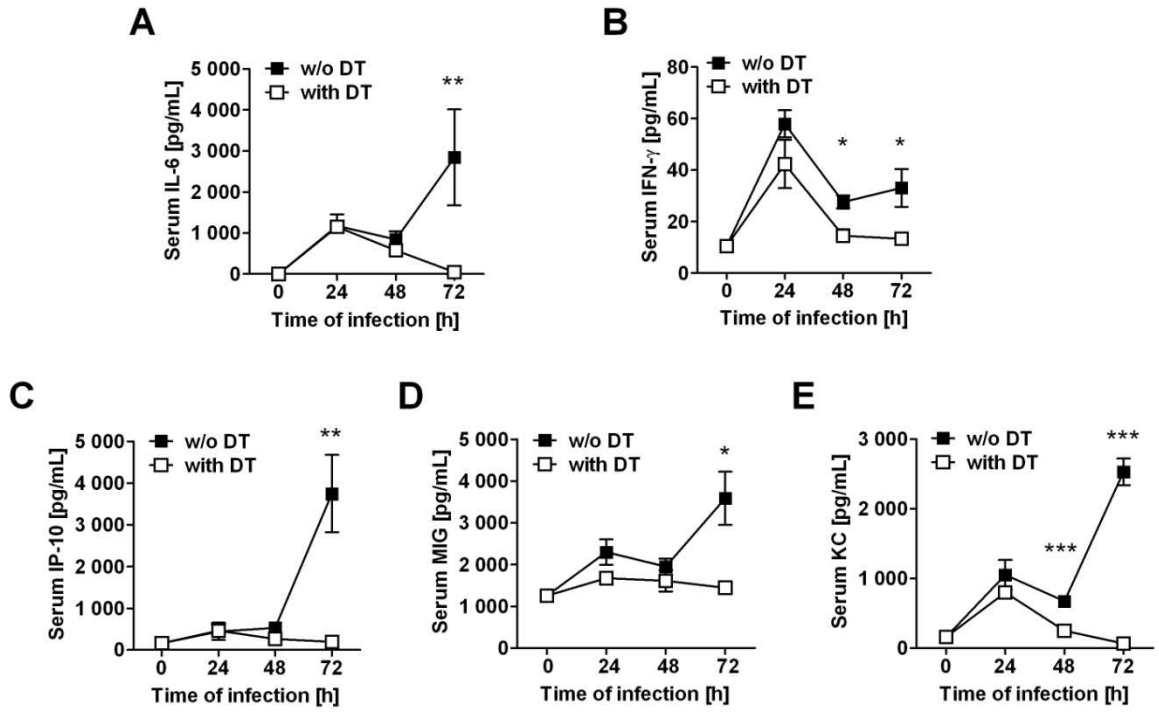


Figure 12: Kinetics of serum cytokines/chemokines in DC-depleted and non-depleted CD11c-DTR chimera mice during the course of *S. pneumoniae* infection. DC-depleted (white symbols) and non-depleted (black symbols) mice were challenged intranasally with $1 \times 10^8$ CFU of *S. pneumoniae* D39 and the levels of IL-6 (A), IFN-γ (B), IP-10(C), MIG (D) and KC (E) in serum were determined by multiplex technology at progressive times after bacterial inoculation. Each symbol represents the mean ± SD of the compilation of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$.

## 4.3. Depletion of DCs does not affect the recruitment of leukocytes into the *S. pneumoniae*-infected lungs

Since depletion of DCs has been shown to have a beneficial effect on the host response to intranasal challenge with *S. pneumoniae*, this raises the question of whether other immune cells compensate for the lack of DCs, resulting in enhanced bacterial clearance. Therefore, the total amount as well as the subset composition of cells recruited into the lungs of CD11c-DTR chimera mice was determined before and during *S. pneumoniae* infection. The total amount of cells recruited into the *S. pneumoniae*-infected lungs was increased and in the same amount in DC-depleted and non-depleted mice (Fig.13A). The analysis of the different subsets of immune cells recruited to the lungs of infected CD11c-DTR chimera mice revealed no significant differences whether DCs were depleted or not. Thus, macrophages (Fig. 13B), $CD4^+$ T cells (Fig. 13C) and $CD8^+$ T cells (Fig. 13D) were recruited in comparable amounts into the lungs of DC-depleted and non-depleted mice.

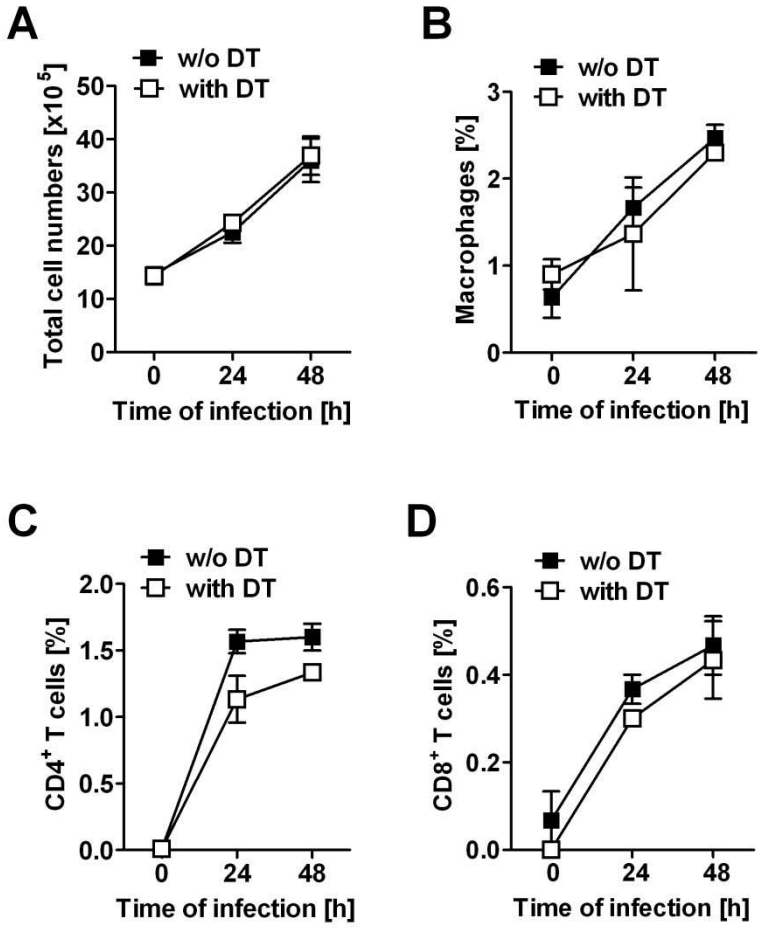


Figure 13: Recruitment of different immune cell populations into the lungs of DC-depleted and non-depleted CD11c-DTR chimera mice during the course of *S. pneumoniae* infection. Cells were counted (A) and the infiltration of macrophages (B), $CD4^+$ T cells (C) and $CD8^+$ T cells (D) into the lungs of DC-depleted (white symbols) and non-depleted (black symbols) CD11c-DTR chimera mice before and at 24 and 48 h after intranasal infection with *S. pneumoniae* D39 was determined by flow cytometry. Each symbol represents the mean ± SD of the compilation of three independent experiments.

Furthermore, monitoring the infiltration of neutrophils into the lungs of CD11c-DTR chimera mice revealed a significant increase in the lungs of uninfected DC-depleted mice (Fig. 14A). In line with this, the levels of the neutrophil-attracting chemokines KC and MIP-2 were significantly increased in uninfected lungs of CD11c-DTR chimera mice after DC depletion (Fig. 14B and 14C). No significant differences were observed in the recruitment of neutrophils (Fig. 14A), levels of KC (Fig. 14B) and levels of MIP-2 (Fig. 14C) in the lungs between *S. pneumoniae*-infected DC-depleted and non-depleted mice. The increase in the amount of neutrophils as a consequence of DC-depletion has also been previously reported [223].

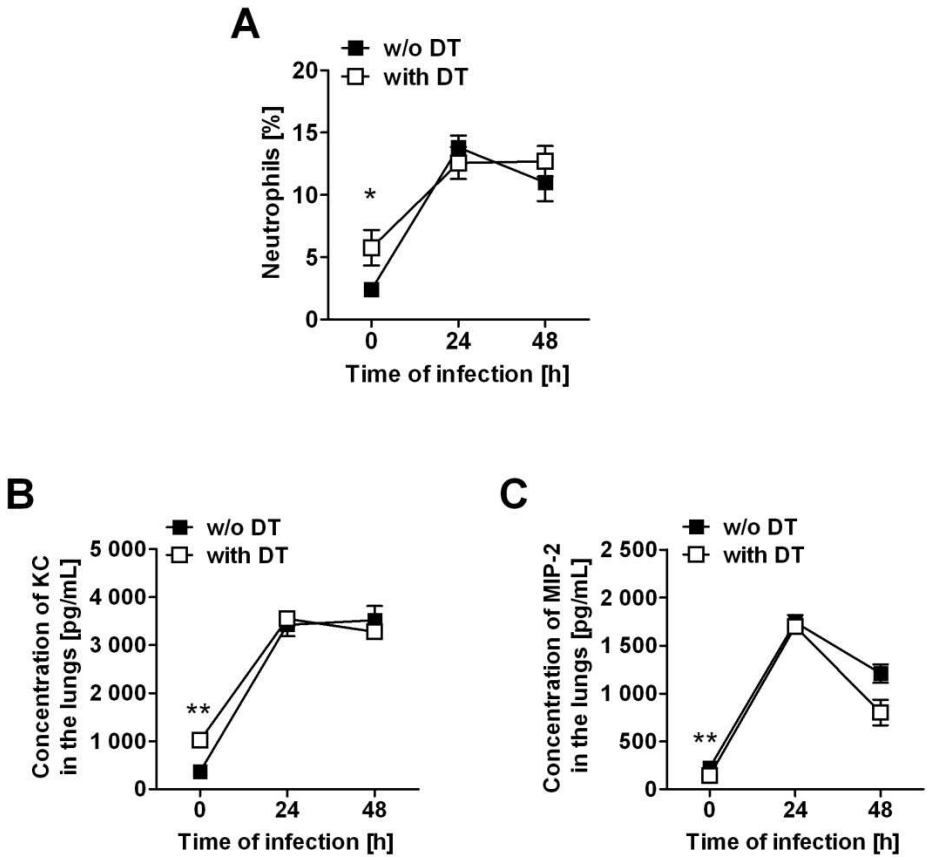


Figure 14: Neutrophil infiltration and neutrophil-attracting chemokine production in the lungs of CD11c-DTR chimera mice in the course of *S. pneumoniae* infection. (A) Infiltration of neutrophils into the lungs of DC-depleted (white symbols) and non-depleted (black symbols) CD11c-DTR chimera mice at progressive time points after intranasal infection with *S. pneumoniae* D39. Concentration of neutrophil-specific chemokines KC (B) and MIP-2 (C) in the lungs of DC-depleted and non-depleted CD11c-CTR chimera mice at progressive time points after inoculation with *S. pneumoniae* D39. Each symbol represents the mean ± SD of the compilation of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$.

Neutrophils play a key role in the front-line of defense against invading pathogens and have indeed been shown to play a major role in the host defense mechanism during bacterial pneumonia [224]. To determine whether the increased amount of neutrophils in the lungs of DC-depleted mice could be responsible for their superior resistance to *S. pneumoniae*, CD11c-DTR chimera mice were either depleted of neutrophils or of both DCs and neutrophils. In both groups, DC-depleted and control mice, the depletion of neutrophils resulted in significantly enhanced bacterial loads in the lungs (Fig. 15A), demonstrating that neutrophils are crucial for efficient clearance of *S. pneumoniae*. However, the amount of pneumococci in the lungs of neutrophil-depleted mice was significantly greater than in neutrophil/DC-depleted animals (Fig. 15A). Whereas all neutrophil-depleted mice exhibited higher bacterial loads in the draining mediastinal lymph nodes, just one half of the

neutrophil/DC-depleted mice had colonized lymph nodes as well as very low amounts of bacteria (Fig. 15B). Furthermore, a significant fraction (75%) of the neutrophils-depleted mice was bacteremic, although none of the neutrophil/DC-depleted animals exhibited pneumococci in the blood (Fig. 15C). The amount of bacteria in the spleen was also significantly higher in neutrophil-depleted than in neutrophil/DC-depleted mice (Fig. 15D). Taken together, these results demonstrate that the improved resistance of DC-depleted CD11c-DTR chimera mice to *S. pneumoniae* was neither depending on a more efficient recruitment of neutrophils nor on other immune cells.

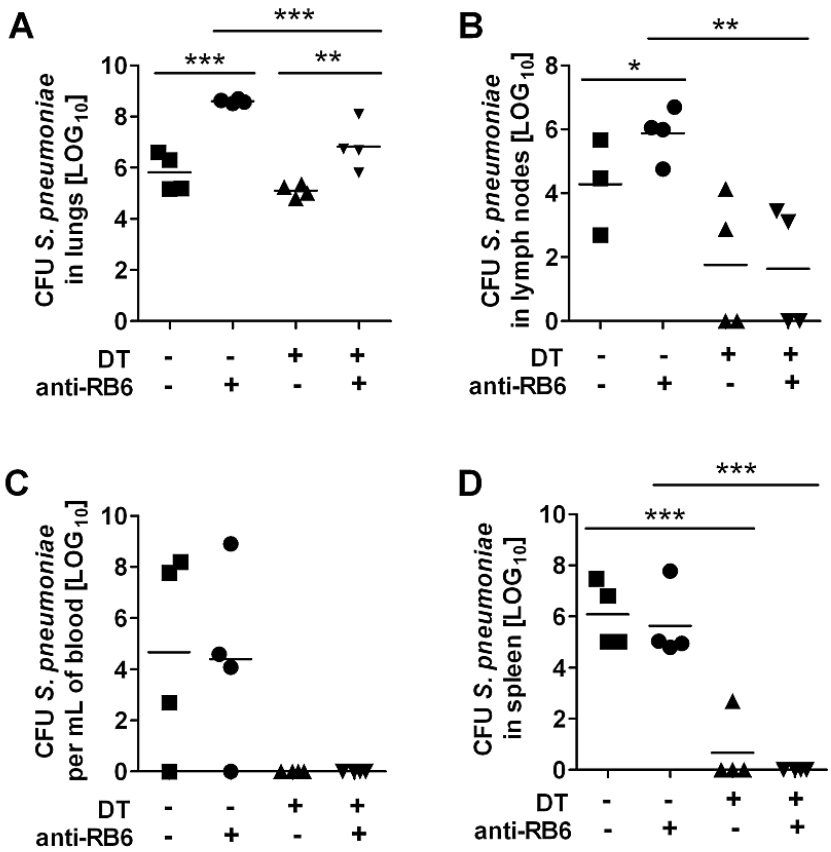


Figure 15: Bacterial burdens in the organs of DC-depleted and non-depleted mice after neutrophil depletion and challenge with *S. pneumoniae*. CD11c-DTR chimera mice were depleted of either DCs, neutrophils or both cell populations and intranasally infected with $1 \times 10^8$ CFU of *S. pneumoniae* D39. Bacterial loads in the lungs (A), mediastinal lymph nodes (B), blood (C) and spleen (D) were determined at 48 h of infection. Each symbol represents an individual mouse. One representative experiment out of three is shown. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

## 4.4. *S. pneumoniae* disseminates from the lungs to the mediastinal lymph nodes in a cell-independent manner

The improved host resistance to *S. pneumoniae* in DC-depleted mice was not due to a more efficient recruitment and bacterial clearance by immune cells. This raises the question of how DCs facilitate disease progression of *S. pneumoniae* infection. As mentioned before, *S. pneumoniae* disseminated from the lungs to the draining lymph nodes to a higher extent than in DC-deficient mice (Fig. 9). Actually, it has been shown that DCs can be manipulated and used as a "Trojan horse" by different pathogens to promote bacterial spreading [225-227]. Therefore, it was investigated whether the pneumococcus uses a similar strategy for its extrapulmonary dissemination. Since expression of CCR7 is crucial for the migration of DCs to the draining lymph nodes [228, 229] the capability of DCs to up-regulate CCR7 in response to *S. pneumoniae* was determined. Flow cytometric analysis of *S. pneumoniae*-infected bone marrow-derived DCs revealed an up-regulation of CCR7 on DCs (Fig. 16A). The degree of CCR7 expression was dependent on the infecting dose of *S. pneumoniae* since increasing levels of CCR7 expression were observed in DCs after exposure to increasing bacterial inocula (Fig. 16A). Thus, *in vitro* infection of DCs with *S. pneumoniae* triggered CCR7 expression on DCs, thereby conferring the DCs with the facility to migrate. The migration of DCs in response to *S. pneumoniae* was also demonstrated *in vivo* after determination of the amount of DCs in the mediastinal lymph nodes of *S. pneumoniae*-infected mice (Fig. 16B). After bacterial inoculation, the number of DCs increased in the lymph nodes of infected mice in a time-dependent manner, whereas no infiltration of DCs could be detected in DC-depleted mice (Fig. 16B). However, the results of the gentamicin protection assays, which allow the discrimination of viable intracellular and extracellular bacteria, indicated that pneumococci were found extracellularly rather than intracellularly within the lymph nodes (Fig. 16C). These results suggest that *S. pneumoniae* disseminated from the lungs and into the regional lymph nodes in a cell-independent manner and that this mode was much more efficient in the presence of DCs.

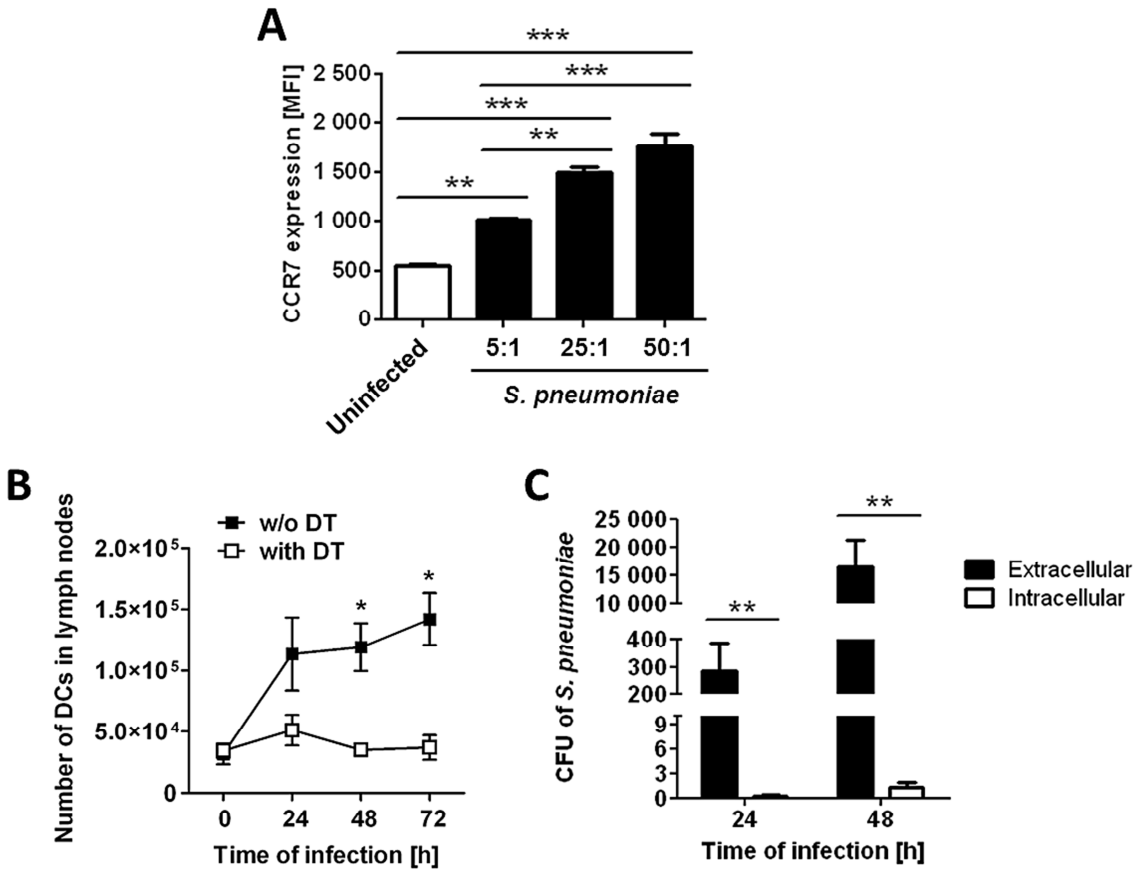


Figure 16: Expression of the chemokine receptor CCR7 on bone marrow-derived DCs and *in vivo* trafficking of DCs to the mediastinal lymph nodes after intranasal infection with *S. pneumoniae*. (A) Bone marrow-derived DCs were either left untreated (white bar) or were infected with different MOI (5:1, 25:1 and 50:1) of *S. pneumoniae* D39. The expression of CCR7 was analyzed by flow cytometry using anti-mouse CCR7 antibodies. (B) Total amount of DCs in the mediastinal lymph nodes of DC-depleted (white symbols) and non-depleted (black symbols) CD11c-DTR chimera mice at progressive time points after intranasal infection with $1 \times 10^8$ CFU of *S. pneumoniae* D39. Lymph node cells were counted, stained with anti-mouse CD11c and anti-mouse CD11b antibodies and analyzed by flow cytometry. Each symbol represents the mean ± SD of three independent experiments. (C) Quantification of viable intracellular (white bars) and extracellular (black bars) *S. pneumoniae* D39 in the mediastinal lymph nodes of infected BALB/c mice at 24 and 48 h after bacterial inoculation. Each bar represents the mean ± SD of triplicate samples. One representative experiment out of three is shown. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

## 4.5. *S. pneumoniae* induces expression of MMP-9 in DCs

When DCs migrate from the site of infection to the draining lymph nodes they have to overcome the barrier of ECMs and basal membranes (BMs). To this end DCs produce enzymes with proteolytic activity such as MMPs. MMPs, especially MMP-9, have the ability to degrade collagen, which is the major component responsible for the barrier function of

ECM and BM. Recent publications [230-233] have reported that some pathogens are able to induce the production and activation of MMP-9 in various cell types to enhance vascular or endothelial permeability thereby promoting their own dissemination. Therefore, the capacity of *S. pneumoniae* to induce the production of MMP-9 by DCs was evaluated. Infection of *in vitro*-cultured bone marrow-derived DCs with *S. pneumoniae* resulted in an up-regulation of the *mmp-9* gene (Fig. 17A). The expression of the *mmp-9* gene increased more than 2-fold in *S. pneumoniae*-infected DCs over the levels observed in uninfected DCs after six hours of infection and up to 3.5-fold at 24 h post infection (Fig. 17A). Not only the expression of MMP-9 was induced in DCs after the challenge with *S. pneumoniae* but also the activation of the proteolytic activity of MMP-9 as demonstrated by the proteolytic activity of culture supernatants originated from *S. pneumoniae*-infected bone marrow-derived DCs at 24 h post infection by gelatin gel zymography (Fig. 17B).

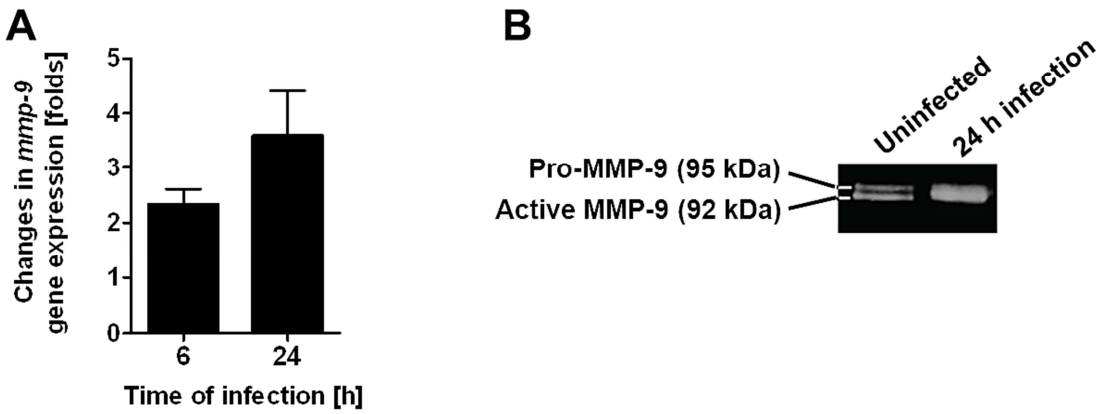


Figure 17: Expression and activation of MMP-9 in bone marrow-derived DCs after exposure to *S. pneumoniae*. (A) Bone marrow-derived DCs were infected with *S. pneumoniae* D39 (MOI 20:1), RNA was extracted from DCs at 6 and 24 h post infection and subjected to real-time RT-PCR for quantification of *mmp-9* gene expression. Results are expressed as fold-change in MMP-9 mRNA of infected DCs over the amount of MMP-9 mRNA in uninfected DCs. Each bar represents the mean of three independent experiments. (B) Representative gelatin gel zymography demonstrating the enhanced activation of MMP-9 in the culture supernatant of *S. pneumoniae*-infected DCs at 24 h post infection compared to the supernatant of uninfected DCs.

The proteolytic activity of the *S. pneumoniae*-stimulated DCs was also demonstrated by the enhanced endothelial permeability in *in vivo* vascular permeability assays. In these experiments, culture supernatant of uninfected and *S. pneumoniae*-infected bone marrow-derived DCs was injected intradermally into the dorsal skin of mice, which have been previously intravenously injected with Evans blue. The photograph displayed in Fig. 18A

shows the areas of the skin where the Evans blue was leaking into the tissue after injection of culture supernatant of either *S. pneumoniae* alone (1), untreated DCs (2) or of *S. pneumoniae*-infected DCs (3). Notably, the area underneath the culture supernatant of *S. pneumoniae*-infected DCs was larger than the areas beneath the supernatant from *S. pneumoniae* or the supernatant from untreated DCs (Fig. 18A). To quantify the degree of the vascular permeability, the areas of Evans blue extravasation were measured, dissected and the amount of Evans blue quantified as described in the Materials and Methods section (section 3.2.13.). The amount of extravasate Evans blue was significantly increased in the area injected with supernatant obtained from *S. pneumoniae*-infected DCs when compared with that of *S. pneumoniae* or supernatant from untreated DCs (Fig. 18B).

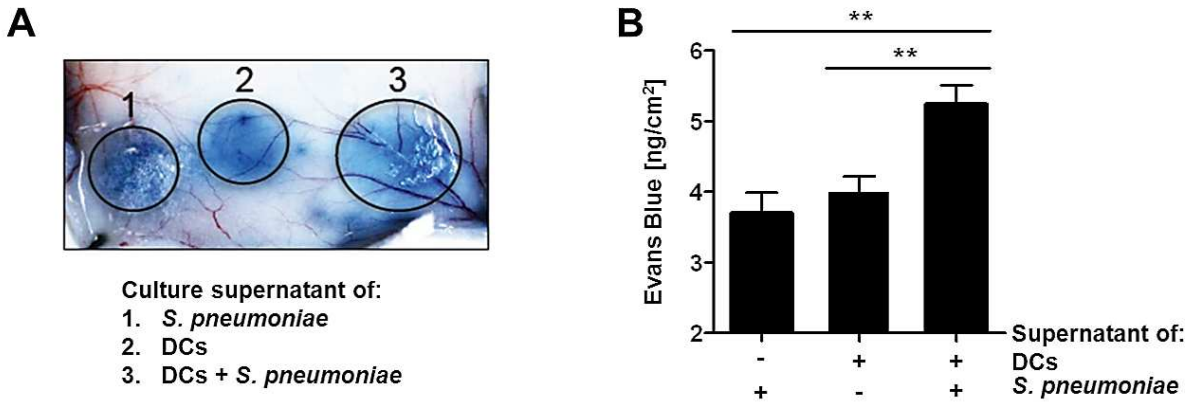


Figure 18: *S. pneumoniae* enhances the induction of vascular permeability by DCs. (A) Vascular permeability induced by the culture supernatant derived from either *S. pneumoniae* D39 (1), untreated DCs (2) or *S. pneumoniae*-infected DCs (3). Supernatant (50 µl) was collected after 24 h, intradermally applied into the skin of Evans blue-treated mice and vascular leakage of Evans blue was visualized 1 h thereafter. A representative experiment out of three is shown. (B) Quantification of Evans blue leakage in the skin of mice after application of supernatant from *S. pneumoniae* D39, untreated DCs or *S. pneumoniae*-infected DCs. Each bar represents the mean ± SD of three independent experiments. **, $p < 0.01$.

## 4.5. Depletion of DCs resulted in marked reduction in the expression of MMP-9 in the lungs of *S. pneumoniae*-infected mice

Although DCs have been demonstrated to produce MMP-9 during *S. pneumoniae* infection *in vitro*, it was still necessary to demonstrate that MMP-9 is up-regulated in the lungs during pneumococcal pneumonia and that DCs are involved in the production of this metalloproteinase during infection. Hence, the expression of the *mmp-9* gene was analyzed in lung homogenates of *S. pneumoniae*-infected BALB/c mice by real-time RT-PCR. As shown

in Fig. 19A, *mmp-9* gene expression was significantly up-regulated in the lungs of *S. pneumoniae*-infected mice with a mean fold-change increase over the uninfected lungs of 4.36 at 24 h and 2.04 at 72 h of infection (Fig. 19A).

To determine the impact of DC depletion on the production of MMP-9 in the lungs during pneumococcal pneumonia, real-time RT-PCR analysis of RNA extracted from lungs of DC-depleted and non-depleted CD11c-DTR chimera mice at 24 h after intranasal challenge with *S. pneumoniae* was performed. Depletion of DCs resulted in significant reduction in the levels of *mmp-9* gene expression in the infected lungs (mean fold-change of 8.23 in non-depleted versus 2.63 fold-change in DC-depleted mice) (Fig. 19B).

As a wide variety of matrix proteins can be also metabolized by the gelatinase MMP-2 [181], the expression of the gene encoding this metalloproteinase was investigated in the lungs of *S. pneumoniae*-infected CD11c-DTR chimera mice. In contrast to MMP-9, gene expression of MMP-2 remained unchanged (mean fold change of 0.94) in the lungs of CD11c-DTR chimera mice after intranasal challenge with S. pneumoniae (Fig. 19C). Furthermore, no difference in the *mmp-2* expression was observed between DC-depleted and non-depleted mice (mean fold-change of 0.94 in non-depleted mice versus 0.76 in DC-depleted CD11c-DTR chimera mice) (Fig. 19C). These results indicate that MMP-2 plays a minor role in the lungs during *S. pneumoniae* infection. Thus, DCs are involved in the production of MMP-9 but not in the production of MMP-2 during pneumococcal pneumonia.

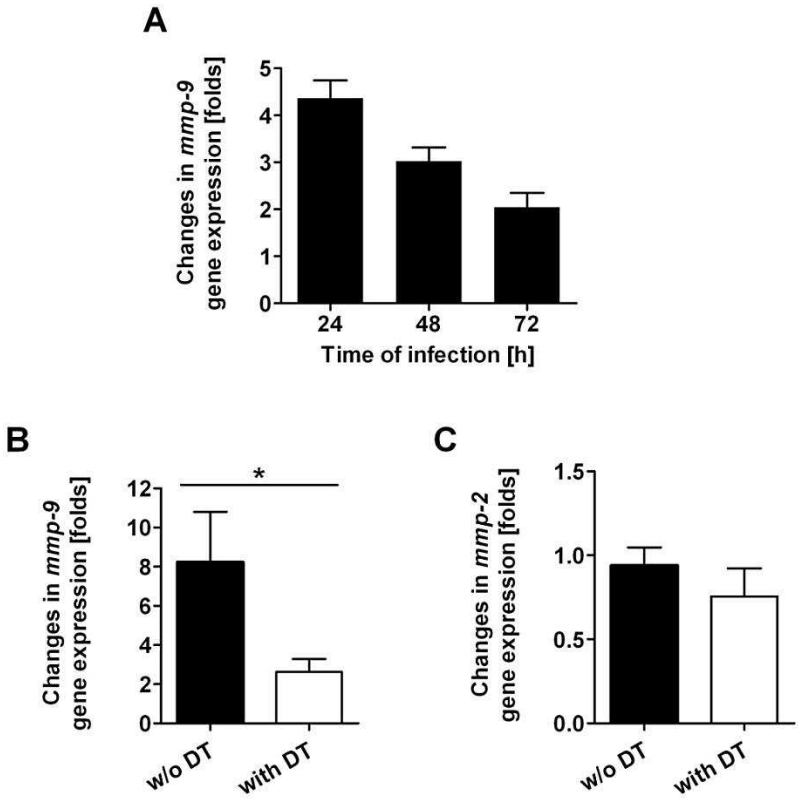


Figure 19: Expression of the *mmp-9* and *mmp-2* genes in the lungs of *S. pneumoniae*-infected mice. (A) Up-regulation of *mmp-9* gene expression in the lungs of BALB/c mice at progressive times after intranasal infection with $1 \times 10^8$ CFU of *S. pneumoniae* D39. (B) Up-regulation of *mmp-9* gene expression in the lungs of DC-depleted (white bar) and non-depleted (black bar) CD11c-DTR chimera mice at 24 h of infection with $1 \times 10^8$ CFU of *S. pneumoniae* D39. (C) Expression of the *mmp-2* gene in the lungs of DC-depleted (white bar) and non-depleted (black bar) CD11c-DTR chimera mice at 24 h of infection with $1 \times 10^8$ CFU of *S. pneumoniae* D39. Results are expressed as fold-change in MMP-9 and MMP-2 mRNA in infected lungs over the amount of MMP-9 and MMP-2 mRNA in uninfected lungs, respectively. Each bar represents the mean value of three independent experiments. *, $p < 0.05$.

## 4.7. *S. pneumoniae* induces partial maturation of bone marrow-derived DCs

To further investigate the response of murine DCs to *S. pneumoniae*, the phenotypic maturation of *S. pneumoniae*-stimulated bone marrow-derived DCs was analyzed by flow cytometry analysis of maturation markers and by detection of pro-inflammatory cytokines in the culture supernatant using ELISA. LPS was used as a positive control for the maturation of DCs. During the maturation process DCs up-regulate the expression of the surface molecules CD40, CD80, CD86 and MHC class II as well as the production of pro-inflammatory cytokines such as IL-6, TNF-α and in particular IL-12 [172]. These molecules are necessary

for efficient T cell activation and induction of adaptive immune responses [234]. *S. pneumoniae* induced a significant up-regulation of the costimulatory molecules CD80 (Fig. 20 and 21B) and CD86 (Fig. 20 and 21C) on murine bone marrow-derived DCs compared to uninfected DCs. In contrast, the maturation markers CD40 (Fig. 20 and 21A) and MHC class II (Fig. 20 and 21D) remained unchanged.

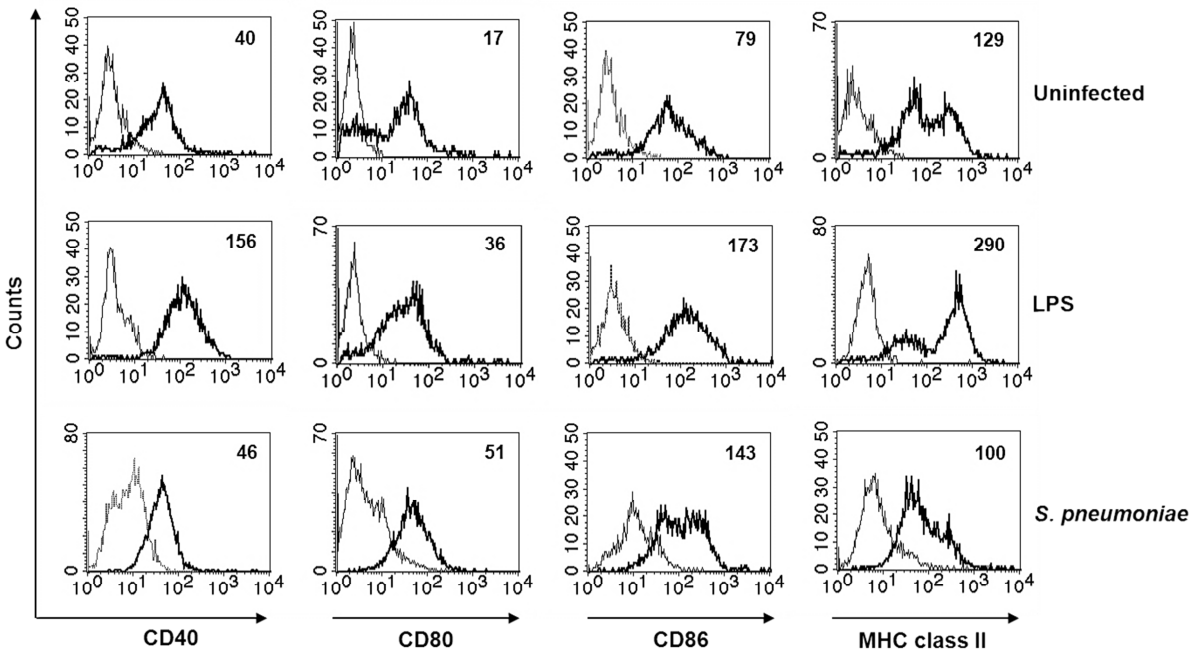


Figure 20: Representative single-parameter histograms showing cell surface expression of maturation markers on bone marrow-derived DCs after challenge with *S. pneumoniae*. Bone marrow-derived DCs were either left untreated, infected with *S. pneumoniae* D39 (MOI 20:1) or treated with 1 µg/mL of LPS. DCs were collected after 24 h and stained for the expression of CD11c as well as for the expression of maturation markers CD40, CD80, CD86 and MHC class II and analyzed by flow cytometry. Histograms depicting the fluorescence intensity of DCs stained with antibodies against specific markers (thick lines) are overlaid on histograms of cells incubated with the isotype control antibody (thin lines). The numbers in each panel indicate the mean fluorescence intensity (MFI) of the specific marker.

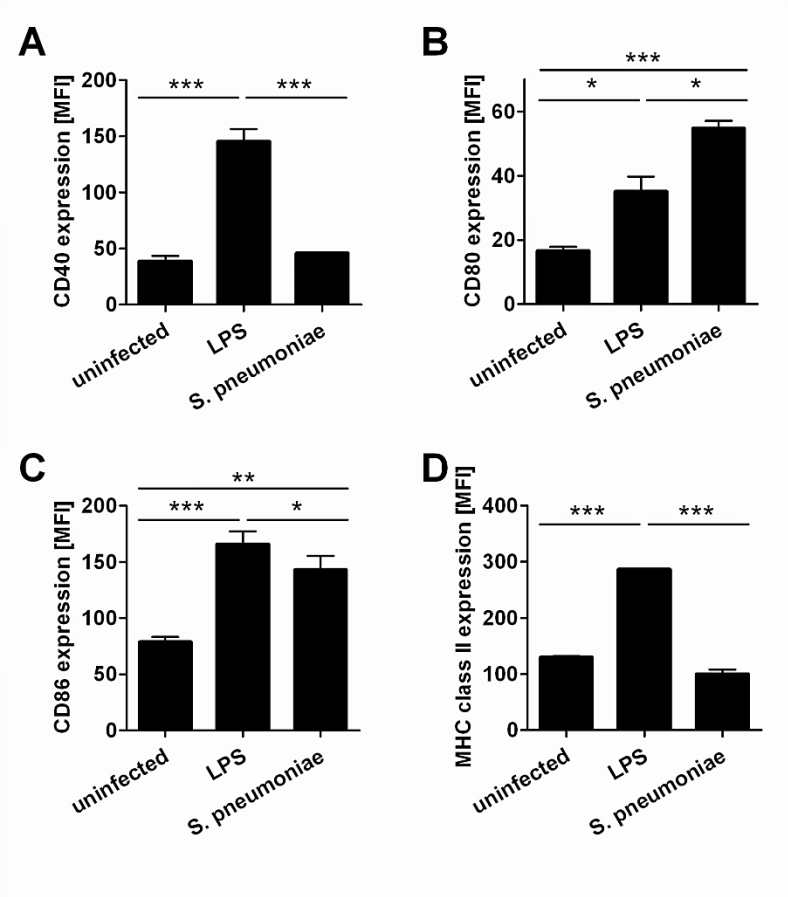


Figure 21: Quantification of cell surface expression of maturation markers on bone marrow-derived DCs in response to *S. pneumoniae* infection. Bone marrow-derived DCs were either left untreated, infected with *S. pneumoniae* D39 (MOI 20:1) or treated with 1 µg/mL of LPS. DCs were collected after 24 h and stained for the expression of CD11c as well as for the expression of maturation markers CD40, CD80, CD86 and MHC class II and analyzed by flow cytometry. MFI of the surface molecules CD40 (A), CD80 (B), CD86 (C) and MHC class II (D) on the above-mentioned DCs are depicted in the graphs. Each bar represents the mean ± SD of triplicates. Data from one representative experiment out of three is shown. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

Regarding the production of cytokines, *S. pneumoniae*-infected DCs produced higher levels of IL-6 and TNF-α than uninfected DCs. However, they did not reach the levels observed in the supernatant of LPS-stimulated DCs (Fig. 22A and 22B). Interestingly, the production of the pro-inflammatory cytokine IL-12 was strongly down-regulated in DCs after exposure to *S. pneumoniae* (Fig. 22C). As it has been previously reported that the pneumolysin of *S. pneumoniae* induces caspase-dependent apoptosis in infected DCs [210], the possibility that the decreased amount of cytokines observed in the supernatant of *S. pneumoniae*-infected DCs is related to the lower viability of DCs was determined. Although infection with *S. pneumoniae* affected the viability of DCs to some extent (Fig. 22E), this cannot account for the reduced levels of the pro-inflammatory cytokines IL-6, TNF-α and IL-12 since the

expression of the chemokine KC was up-regulated to a similar extent in infected DCs compared to LPS-stimulated DCs (Fig. 22D), albeit LPS did not affect DC viability. Taken together, this data indicates that *S. pneumoniae* impaired full maturation of DCs. Since DCs play an extraordinary role as central orchestrators of innate and adaptive immune responses, partial maturation might strongly influence the host response to *S. pneumoniae*.

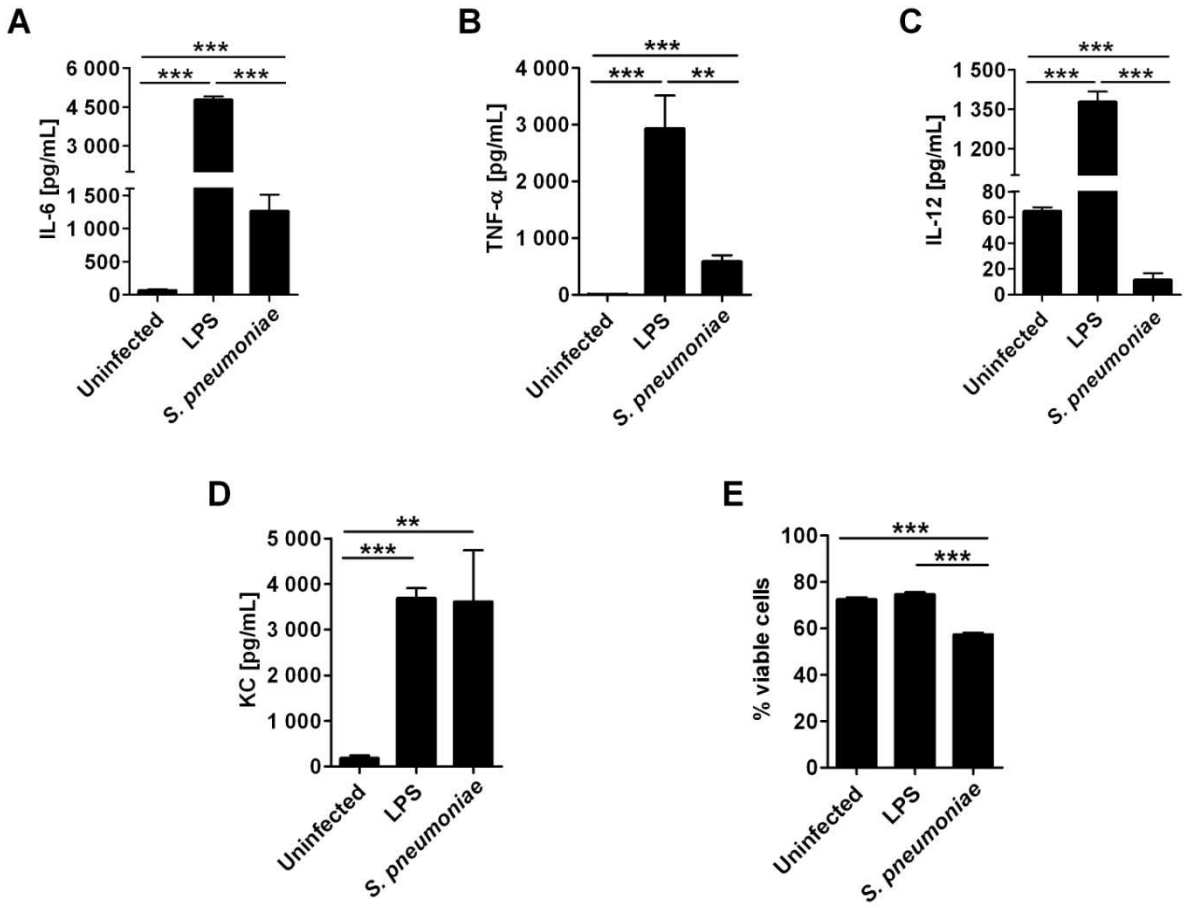


Figure 22: Production of pro-inflammatory cytokines/chemokines by bone marrow-derived DCs after challenge with *S. pneumoniae*. Bone marrow-derived DCs were either infected with *S. pneumoniae* D39 (MOI 20:1), treated with 1 µg/mL of LPS or left untreated. Culture supernatants were collected after 24 h and the levels of IL-6 (A), TNF-α (B), IL-12 (C) and KC (D) were determined by ELISA. (E) DC viability was determined by analyzing the percentage of cells negative for Annexin V and PI using flow cytometry. Each bar represents the mean ± SD of triplicates. Data from one representative experiment out of three is shown. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

## 4.8. Inhibition of DC maturation is caused by secreted molecules of *S. pneumoniae*

Since the *S. pneumoniae*-infected DCs fail to fully mature, the question arises whether the bacterial stimulus was too weak to induce DC full maturation or whether *S. pneumoniae* actively inhibited the maturation process. The fact that IL-12, produced to a certain extent by immature DCs, was completely down-regulated by DCs after exposure to *S. pneumoniae* indicated an active inhibitory effect (Fig. 22C). Since pneumolysin has been reported to inhibit the maturation of human DCs [210], it was investigated whether the maturation of murine DCs was also partially inhibited by *S. pneumoniae*. For this purpose, LPS-stimulated bone marrow-derived DCs, which had high expression of CD40 (Fig. 21A and 23A) and produced high levels of IL-12 (Fig. 22C and 23B) were cultured in the presence of culture supernatant isolated from *S. pneumoniae*. Flow cytometry analysis of CD40 expression on LPS/*S. pneumoniae*-treated DCs revealed a significant decrease in the level of CD40 expression when compared to LPS-stimulated DCs (Fig. 23A). Furthermore, LPS/*S. pneumoniae*-treated DCs showed a strong reduction of IL-12 production compared to uninfected or LPS-treated DCs (Fig. 23B). These results indicate on the one hand that *S. pneumoniae* actively inhibits certain pathways of DC maturation and, on the other hand, that this inhibitory effect is mediated by a released bacterial constituent rather than by direct cell-cell contact.

To further investigate if pneumolysin was involved in the inhibition of DC maturation, murine bone marrow-derived DCs were infected with the pneumolysin-deficient mutant D39Δ*ply* and the expression of CD40 (Fig. 23A) and production of IL-12 (Fig. 23B) were determined and compared with that of DCs infected with the wild-type strain. D39Δ*ply* did not induce up-regulation of CD40 in DCs (Fig. 23A), indicating that the inhibition of this maturation pathway was independent of the presence/absence of pneumolysin. However, D39Δ*ply* did not inhibit the production of IL-12 by DCs as opposed to the strong inhibition exerted by the wild-type strain (Fig. 23B). This result implicates pneumolysin in the inhibition of IL-12 production.

Taken together, these data demonstrate that pneumococcal pneumolysin is involved in the inhibition of murine DC maturation. Nevertheless, it cannot be excluded that additional *S. pneumoniae* molecule(s) are involved in this process as well. Further studies are therefore required to identify the mechanisms of the inhibition process.

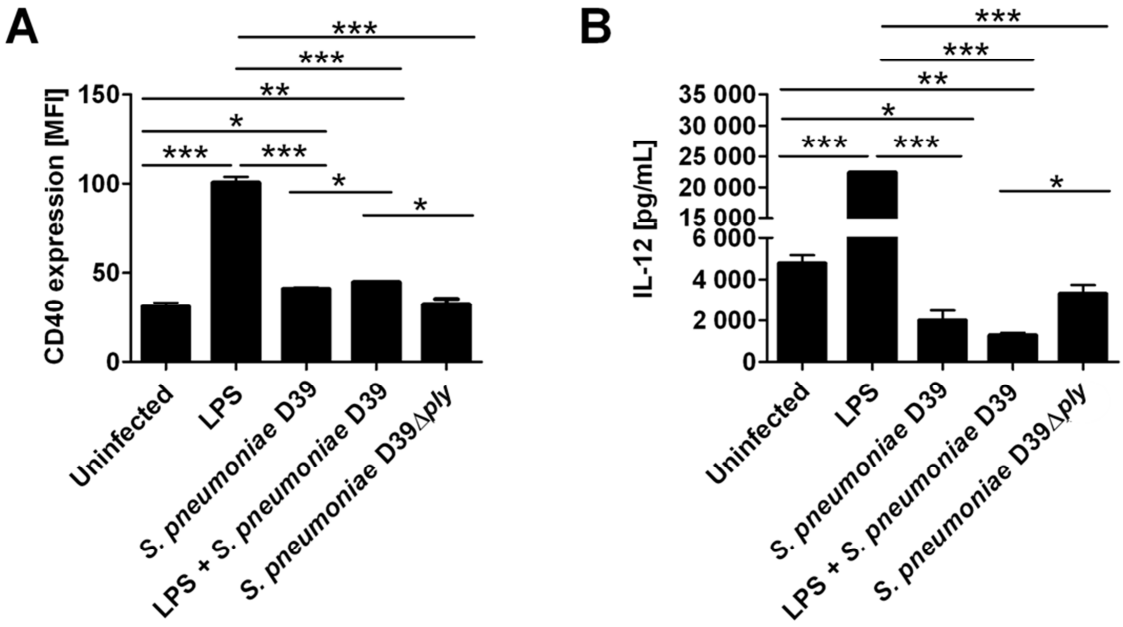


Figure 23: Expression of CD40 and IL-12 by DCs after exposure to LPS, *S. pneumoniae* D39, LPS and *S. pneumoniae* D39 or pneumolysin-deficient *S. pneumoniae* (D39Δ*ply*). Bone marrow-derived DCs were either left untreated, infected with *S. pneumoniae* D39 or *S. pneumoniae* D39Δ*ply* (MOI 20:1), treated with 1 µg/mL of LPS or incubated simultaneously with LPS and culture supernatant of *S. pneumoniae* D39. DCs and culture supernatants were collected after 24 h. Cells were stained for the expression of CD11c as well as for the expression of maturation marker CD40 and analyzed by flow cytometry. MFI of the surface molecule CD40 on the above-mentioned DCs is depicted (A). IL-12 was determined by ELISA in the culture supernatants of the differential treated DCs (B). Each bar represents the mean ± SD of triplicates. Data from one representative experiment out of two is shown. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

# 5. Discussion

## 5.1. Depletion of DCs renders mice more resistant to respiratory challenge with *S. pneumoniae*

*S. pneumoniae* is an important pathogen and the cause of invasive diseases such as sepsis, meningitis, and pneumonia [4]. The treatment of pneumococcal infections is complicated by the worldwide emergence of antibiotic-resistant strains with limited treatment options [98, 99]. Although, the current available vaccine is effective at conferring protection against invasive disease caused by the vaccine-serotype strains, vaccine coverage is limited, and replacement by non-vaccine serotypes is a serious problem [12]. Consequently, *S. pneumoniae* remains still an important public health problem being responsible for more than 1 million deaths every year [235]. New therapeutic strategies are therefore required to combat this pathogen. In this regard, therapeutic strategies based on the modulation or fine-tuning of the host immune response to kill the pathogen more efficiently may provide a new option in the treatment of pneumococcal infections. However, the development of such strategies requires a better understanding of the host immune response to *S. pneumoniae*. Because DCs are among the first immune cells which come in contact with invading pathogens and who are critical for inducing and shaping the pathogen-specific immune responses [236], the objective of this thesis was to elucidate the role played by DCs during *S. pneumoniae* infection.

A mouse model (CD11c-DTR chimera mice) that allows the conditional and transient depletion of $CD11c^+$ cells, in particular DCs, was used to define the impact of DCs in host defense against respiratory infection with *S. pneumoniae*. Remarkably, the depletion of DCs rendered CD11c-DTR chimera mice more resistant to pneumococcal infection, demonstrated by reduced bacterial loads in the organs, delayed bacterial dissemination and reduced cytokine and chemokine levels in the serum when compared with non-depleted mice (Fig. 9 and Fig. 12). As not only DCs but also alveolar macrophages are depleted in the CD11c-DTR chimera mice [222], it was not possible to ascribe the increased resistance of mice to *S. pneumoniae* solely to the depletion of DCs. However, based on the report of Knapp *et al.* [145] demonstrating that *in vivo* depletion of alveolar macrophages alone by intranasal instillation of liposomal dichloromethylene-bisphosphonate prior to infection with *S. pneumoniae* significantly increased the mortality of infected animals [145], it was conceivable to attribute the enhanced resistance of mice to *S. pneumoniae* infection exclusively to the depletion of

DCs. Furthermore, the results of this work are consistent with the previous study of Winter *et al*. [237] in which increased amount of DCs in the blood and lungs of mice after administration of FMS-like tyrosine kinase 3 ligand (Flt3L) resulted in enhanced mortality and morbidity of mice after intratracheal instillation of *S. pneumoniae*. Together, these observations indicate that DCs might have an adverse rather than a beneficial effect on the outcome of pneumococcal pneumonia. Moreover, the improved resistance to infection with *S. pneumoniae* was neither strain specific (Fig. 10) nor dependent on a side effect of DT treatment *per se* (Fig. 11).

The results of this thesis also demonstrated that the enhanced resistance to *S. pneumoniae* of DC-depleted mice was not mediated by an altered recruitment of inflammatory cells into the infected lungs. It has been previously reported that DC depletion in CD11c-DTR mice after treatment with DT resulted in neutrophilia and enhanced recruitment of neutrophils to the periphery [223]. Furthermore, in the recent study of Autenrieth *et al.* [238] it was shown that depletion of DCs improved clearance of *Yersinia enterocolitica* by enhancing the recruitment of neutrophils to the infected tissue as well as by increasing the production of reactive oxygen species (ROS) and thereby the bactericidal activity of these phagocytic cells. This was not the case for *S. pneumoniae* infection since although DT treatment alone resulted in increased neutrophil infiltration into the lungs of uninfected CD11c-DTR chimera mice (Fig. 14), DC-depleted mice were still more resistant to *S. pneumoniae* than non-depleted mice in the absence of neutrophils (Fig. 15). This can be explained by the fact that ROS do not play a role in controlling pneumococcal infection neither in the lungs nor in the brain and mice deficient in enzymes involved in ROS production were not inferior in the clearance of *S. pneumoniae* compared to control mice [239, 240].

## 5.2. S. pneumoniae stimulates production and activation of MMP-9 by DCs that might facilitate bacterial dissemination

After pathogen recognition, respiratory DCs generally start a maturation program that involves the migration from the infected lungs to the mediastinal lymph nodes. During infection with *S. pneumoniae* the amount of DCs in the mediastinal lymph nodes increased with the progression of infection confirming the migratory capacity of these cells (Fig. 16B). The observation in this study that pneumococci disseminated to the local lymph nodes to a greater extent in the presence of DCs (Fig. 9B) might suggest that these bacteria could take advantage of the migratory capacity of DCs to promote their own dissemination. In this

regard, several pathogens including *Salmonella typhimurium*, *Listeria monocytogenes* and *Mycobacterium tuberculosis*, have been shown to enter DCs and use them as a "Trojan horse" to disseminate within the host [225, 241-243]. However, the fact that a high proportion of pneumococci within the lymphoid tissue were extracellular (Fig. 16C) suggests that *S. pneumoniae* disseminated from the lungs to the lymph nodes in a cell-free manner and not within eukaryotic cells.

To migrate into the lymph nodes, *S. pneumoniae* has to cross the ECM and BM. In this regard, a role for the plasminogen/plasmin system in facilitating the transmigration of pneumococci through connective tissues such as the ECM has been described [91]. This involves the adherence of *S. pneumoniae* to glycoproteins of the ECM via PfbA and PavA and immobilization of plasminogen on the outer surface of *S. pneumoniae* by the plasminogen receptors α-enolase, GAPDH or CBPE [93, 94, 244]. In particular, the α-enolase seems to play a major role in the recruitment of plasminogen since a mutant lacking the binding site for plasminogen was barely capable to transmigrate [91]. Plasminogen immobilized on the bacterial surface is subsequently converted into plasmin by host-derived activators. Plasmin degrades the ECM components laminin and fibronectin, but is poorly active against native collagens and elastin [245]. Furthermore, plasmin can specifically activate host-derived gelatinases such as MMP-2 and MMP-9 [246, 247]. These MMPs degrade laminin and fibronectin, but are also highly active against collagen [246], which is the major component responsible for the barrier function of the ECM [96]. Under physiological conditions, MMPs contribute to the controlled degradation and remodeling of the ECM [248, 249], which is a crucial event for the migration of DCs [250-252]. On the other hand, an increased expression of gelatinases has been shown to correlate with the severity of diseases such as multiple sclerosis [253], intracerebral hemorrhage [254], bacterial meningitis [255-257] and tuberculosis [258]. There is also evidence that MMP-9 contributes to the severity of pneumococcal pneumonia [259]. Furthermore, Yasuda *et al.* [260] reported improved airway resistance to *S. pneumoniae* after suppression of MMP-9. The results of this study demonstrated that MMP-9 activity was up-regulated in the lungs of mice during pneumococcal pneumonia as well as on DCs after exposure to *S. pneumoniae.* Furthermore, depletion of DCs correlated with a significant decrease in MMP-9 expression in the lungs of *S. pneumoniae*-infected mice, which supports that DCs are either a direct source of MMP-9 during pneumococcal pneumonia or that they modulate MMP-9 production by other cell types. The expression of other MMPs such as MMP-2 was unchanged in the lungs of mice after intranasal challenge with *S. pneumoniae* (Fig. 19C).

MMPs are secreted in a latent pro-form into the extracellular space which has to be activated by proteolytic cleavage [261]. In addition to the expression of MMP-9, *S. pneumoniae* stimulated the activation of pro-MMP-9 produced by bone marrow-derived DCs (Fig. 17B). Although it has been reported that *S. pneumoniae* produces a zinc metalloproteinase ZmpC that directly activates MMP-9 *in vitro*, the gene encoding ZmpC is not present in the strain of *S. pneumoniae* used in this study (D39) [95]. Hence, activation of MMP-9 by *S. pneumoniae* seems to be mediated by a not yet identified bacterial virulence factor.

Based on all these observations, it can be hypothesized that the increased production of MMP-9 by pulmonary DCs during pneumococcal pneumonia resulted in a more efficient extracellular matrix breakdown thereby facilitating *S. pneumoniae* to cross biological barriers and to disseminate. In this regard, selective blockade of MMPs might be beneficial for the outcome of the infection and therefore provide a novel adjunct to antibiotic-based therapeutic intervention in pneumococcal pneumonia. Promising studies demonstrated that MMP activity can be inhibited with a hydroxamic acid-based inhibitor which reduced mortality of pneumococcal meningitis in a rat model [262] or by chemically modified tetracyclines that reduced mortality of rats in a sepsis model [263]. However, since MMPs are host-derived enzymes, the possibility that MMP inhibitors might block normal physiological functions with subsequent side effects should be carefully considered.

### 5.3. *S. pneumoniae* inhibits the maturation of bone marrow-derived DCs

The maturation of DCs is crucial for the orchestration of innate immune responses and the initiation of adaptive immunity. *S. pneumoniae* induced only partial maturation of murine bone marrow-derived DCs *in vitro* characterized by high surface expression of CD80 and CD86, diminished expression of CD40 and MHC class II as well as a complete abrogation of IL-12 (Fig. 21 and Fig. 22). Since these molecules, particularly IL-12, are needed to prime naive T cells to become effector T cells [264], impairing DC maturation might be an advantageous mechanism of *S. pneumoniae* to escape the host immune response. Several bacterial pathogens have been identified which have developed mechanisms to subvert DC function. *Mycobacterium tuberculosis* interferes with TLR signaling via the C-type lectin DC-SIGN, blocking DC maturation and IL-12 production [265]. Instead, DCs secrete IL-10, which directs the immune response towards immune suppression that contributes to the chronic carriage of this pathogen. Similarly, *Bordetella pertussis*-infected DCs secrete IL-10 and activate Treg cells that suppress a protective immune response and enhance colonization

of the lower respiratory tract [174, 266]. In addition, several studies have shown that *Salmonella typhimurium* induces secretion of pro-inflammatory cytokines [267, 268], but it is able to block MHC class II antigen presentation in bone marrow-derived DCs [269, 270]. Another bacterial pathogen, *Francisella tularensis*, which induces phenotypic maturation of DCs, has been shown to inhibit secretion of pro-inflammatory cytokines such as TNF-α while eliciting production of immunosuppressive cytokines that facilitate pulmonary infection [271].

In addition, results of this study indicate that *S. pneumoniae* is also capable to inhibit LPS-driven maturation of murine bone marrow-derived DCs (Fig. 23). Such inhibitory capacity has also been described for the human cytomegalovirus [272] and the filamentous hemagglutinin of *Bordetella pertussis* that inhibit LPS-induced IL-12 and inflammatory chemokine production by DCs [174].

Regarding the *S. pneumoniae* factors involved in the inhibition of IL-12 production, Littmann *et al.* [210] demonstrated that the cholesterol-dependent cytotoxin pneumolysin inhibits the activation of human DCs. *S. pneumoniae* impaired the expression of co-stimulatory molecules CD80 and CD86 and inhibited the production of IL-12 by human DCs. Furthermore, infection of human DCs with a pneumolysin-deficient mutant caused significantly less severe inhibition of DC maturation than the wild-type strain. Similar results have been revealed in this study using murine DCs. The lack of CD40 up-regulation by DCs was independent of the presence or absence of pneumolysin. In contrast, the pneumolysin-deficient mutant D39Δ*ply* failed to inhibit the IL-12 production by DCs confirming the direct involvement of this cytotoxin in the IL-12 inhibition.

In summary, the current study provides direct *in vivo* evidence that *S. pneumoniae* exploits DCs to disseminate from the initial site of infection. Furthermore, *in vitro* data demonstrate that this pathogen modulates DC functions by interfering with the maturation process. Therefore, DCs might provide an interesting therapeutic target during pneumococcal pneumonia to prevent or delay extrapulmonary bacterial dissemination as well as to induce protective immune responses.

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# 7. Appendix

## 7.1. List of abbreviations

**Table 5: Abbreviations**

| | |
|---|---|
| °C | degree Celsius |
| 3´ | three prime end |
| 5´ | five prime end |
| 7-AAD | 7-amino-actinomycin |
| Ab | antibody |
| ACK | ammonium-chloride-potassium |
| *ad* | *ad* (Latin ‘up to’) |
| ADP | adenosindiphosphat |
| AOM | acute otitis media |
| AP-1 | activator protein 1 |
| APC | antigen presenting cell |
| *Aq. dest* | *aqua destillata* (Latin ‘distilled water’) |
| BM | basal membrane |
| C3 | complement component 3 |
| CBP | choline-binding protein |
| CCL | chemokine (C-C-motif) ligand |
| CCR | chemokine (C-C-motif) receptor |
| CD | cluster of differentiation |
| cDNA | complementary deoxyribonucleic acid |
| CFU | colony forming units |
| $CO_2$ | carbon dioxide |
| COPD | chronic obstructive pulmonary disease |
| CpG | cytosine-phosphate-guanosine |
| CXCL | chemokine (C-X-C motif) ligand |
| Cya | adenylate cyclase toxin |
| DC | dendritic cell |
| $ddH_2O$ | autoclaved MilliQ water |
| DMSO | Dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |

| | |
|---|---|
| DT | diphtheria toxin |
| DTR | diphtheria toxin receptor |
| ECM | extracellular matrix |
| EDTA | ethylenediaminetetraacetic acid |
| EF | elongation factor |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| EndA | endonuclease A |
| *et al* | *et alii* (Latin 'and others') |
| FACS | fluorescence-activated cell sorting |
| Fc | fragment, crystallisable |
| FCS | fetal calf serum |
| FHA | filamentous hemagglutinin |
| Fig | figure |
| FITC | Fluorescein isothiocyanat |
| Flt3L | FMS-like tyrosine kinase 3 ligand |
| FMS | receptor to the macrophage colony-stimulating factor |
| g | gram |
| g | g-force |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| h | hour |
| $H_2O$ | water |
| IFN | interferon |
| IL | interleukin |
| IP | interferon gamma-induced protein |
| KC | keratinocyte chemoattractant |
| kg | kilogram |
| L | liter |
| log | logarithm |
| LPS | lipopolysaccaride |
| LTA | lipoteichoic acid |
| M | molarity |
| MARCO | macrophage receptor with collagenous structure |
| MFI | mean fluorescence intensity |

| | |
|---|---|
| MHC | major histocompatibility complex |
| MIG | monokine induced by gamma-interferon |
| min | minute |
| MIP | macrophage inflammatory protein |
| mL | milliliter |
| mM | millimol |
| MMP | matrix metalloproteinase |
| MOI | multiplicity of infection |
| mRNA | messenger ribonucleic acid |
| NET | neutrophil extracellular traps |
| NF-κB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| ng | nanogram |
| NK | natural killer |
| nm | nanometer |
| NOD | non-obese diabetic |
| ø | diameter |
| $OD_{600}$ | optical density at 600 nm |
| P value | probability value |
| PAFR | platelet-activating factor receptor |
| Pav | pneumococcal adherence and virulence factor |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PE | phycoerythrin |
| Pfb | plasmin- and fibronectin-binding protein |
| pH | *potentia Hydrogenii* |
| *ply* | gene encoding pneumolysin |
| Ppm | putative proteinase maturation protein |
| PPV | pneumococcal polysaccharide vaccine |
| PRR | pattern recognition receptor |
| Psa | pneumococcal surface adhesin |
| Psp | pneumococcal surface protein |
| PVC | polysaccharide conjugated vaccine |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |

| | |
|---|---|
| rpm | rotations per minute |
| RPMI | roswell park memorial institute medium |
| RT | room temperature |
| RT-PCR | reverse transcription polymerase chain reaction |
| SD | standard deviation |
| SDS | sodium dodecyl sulphate |
| sec | second |
| SIGN-R1 | specific intracellular adhesion molecule-3 grabbing nonintegrin homolog-related 1 |
| Slr | streptococcal lipoprotein rotamase |
| TA | teichonic acid |
| TAE | Tris-acetate-EDTA |
| TCR | T cell receptor |
| TGF | transforming growth factor |
| $T_H$ | T helper |
| THY | Todd-Hewitt Broth with yeast |
| TIMP | tissue inhibitors of matrix metalloproteinases |
| TLR | toll-like receptor |
| TMB | 3,3′,5,5′-tetramethylbenzidine |
| TNF | tumor necrosis factor |
| Treg | regulatory T cell |
| Tween-20 | Polyoxyethylene Sorbitan Monolaurate |
| U | units |
| v/v | volume/volume |
| w/o | without |
| w/v | weight/volume |
| Zmp | zinc metalloproteinase |
| α | alpha |
| β | beta |
| γ | gamma |
| δ | delta |
| κ | kappa |
| μ | micro |

## 7.2. List of figures

## 7.3. List of tables

## 7.4. Acknowledgement

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